MOLECULAR CHARACTERIZATION OF PUTATIVE MESENCHYMAL PROGENITOR CELLS FROM EQUINE BONE MARROW ASPIRATES

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Bone marrow contains many cell types including hematopoietic cells and their precursors, adipocytes, endothelial cells, and osteocytes. There are also cells with reported capacity to differentiate into bone, cartilage, and other tissues. Their descriptive terminology varies and includes mesenchymal stem cell (MSC), stromal stem cell (SSC), and mesenchymal progenitor cell (MPC), used herein. The cell-surface phenotypes of cultured MPCs have been described for humans and other species. However, there is no consensus on their phenotype from uncultured bone marrow mononuclear cells (BMMNC) and poor understanding of their phenotypic changes during culture. These issues complicate clinical use of MPCs in cell-based therapies as extended periods of culture (typically 4-6 weeks) are required to purify and expand cell numbers, making the original phenotype unclear and delaying treatment. To address this problem, my experiments were designed to study the phenotypic changes that occur in equine BMMNCs from isolation through one month culture. Flow cytometry and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were used to analyze cell-surface molecules. Results demonstrated numerous dynamic changes in BMMNC phenotype. Next, cell sorting experiments were performed to determine if phenotypic changes during early culture could be exploited for MPC colony enrichment. Magnetic activated cell sorting (MACS) was used to separate adherent BMMNCs based on their expression of cluster of differentiation (CD) marker CD14. BMMNCs were separated into three groups; CD14 positive, CD14 negative, or unsorted. Flow cytometry and RT-qPCR were used

to evaluate sorting efficiency and compare groups over time. At day seven, cells positively selected for CD14 were significantly more likely to form colonies than both unsorted and negatively selected cells ($P \leq 0.005$). Further, MPCs maintained low levels of CD14 expression in long-term culture and upregulated expression in response to lipopolysaccharide. These findings were surprising because by definition, MPCs are thought to be non-hematopoietic because they lack expression of specific hematopoietic molecules such as CD14. Only certain lineages of hematopoietic cells are known to express CD14. Results of my studies support that equine MPCs express and are enriched by CD14, suggesting they have either been misclassified, or may represent a differentiated descendant of a hematopoietic cell.

BIOGRAPHICAL SKETCH

Catherine H. Radcliffe grew up in Wabasha, Minnesota, the second of seven children of John and Darla Taubel. She was a 1993 graduate of Wabasha-Kellogg High School. Following graduation, she attended Saint Mary's University of Minnesota for the next three years, majoring in both Chemistry and Biology. In 1996, she was offered early admittance to the University of Minnesota College of Veterinary Medicine and subsequently relocated to Saint Paul, Minnesota. In 1998, she received her Bachelor's degree in Animal Science and in 2000, her Doctor of Veterinary Medicine degree from the University of Minnesota. The next year she completed a Large Animal Medical and Surgical Internship at the Ontario Veterinary College, University of Guelph, Guelph, Ontario. In 2001, she served as a Large Animal Surgery Resident followed by an appointment as a Professional Assistant in Large Animal Medicine at the Ontario Veterinary College. Her son, Mo, was also born during that time. In 2002, she moved to Ithaca, NY and began a Residency in Large Animal Surgery at the College of Veterinary Medicine, Cornell University. Upon completion of her residency, she began her doctoral research training in the laboratory of Dr. Lisa A. Fortier in the Graduate Field of Comparative Biomedical Sciences, Department of Clinical Sciences. In 2007, Catherine became a Diplomate of the American College of Veterinary Surgeons as a Large Animal Surgical Specialist. Her research focus is the cell surface proteins of equine bone marrow cells and how cell surface molecules can be used to characterized and isolate the putative mesenchymal progenitor cell population.

This dissertation is dedicated to all the members of my family. You are my past, present and future-the foundation of who I am.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APN	Aminopeptidase N
bFGF	Basic fibroblastic growth factor
BMMNC	Bone marrow mononuclear cells
CD	Cluster of differentiation
CD14	CD14 epitope, lipopolysaccharide receptor
CFU	Colony forming unit
C_T	Cycle threshold
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FABP4	Fatty acid binding protein 4
FACS-Buffer	Fluorescent activated cell sorting buffer
FAM	6-carboxyfluorescin
FL1	Fluorescence channel 1
FL2	Fluorescence channel 2
FSC	Forward scatter
G0	Resting (quiescent) phase of cell cycle
G1	The first phase of interphase during the cell cycle
G2	The gap between DNA synthesis and mitosis in the cell cycle
GFP	Green fluorescent protein
HBSS	Hanks balanced salt solution
H-CAM	Homing-associated cell adhesion molecule

^{hi}	High mean fluorescence intensity
HLDA	Human leukocyte differentiation antigen
HSC	Hematopoietic stem cell
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISCT	International Society for Cellular Therapy
ISSCR	The International Society for Stem Cell Research
ITS+1	1x insulin/transferrin/selenium
LBP	Lipopolysaccharide-binding protein
LCA	Leukocyte common antigen
LFA-1	Lymphocyte function-associated antigen-1
^{lo}	Low mean fluorescence intensity
LPS	Lipopolysaccharide
LPS-R	Lipopolysaccharide receptor (also known as “CD14”)
LY96	Lymphocyte antigen 96 gene
M	The mitosis phases of the cell cycle
Mac-1	Macrophage-1 antigen
MACS	Magnetic activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
^{mod}	Moderate mean fluorescence intensity
MPC	Mesenchymal progenitor cell
MSC	Mesenchymal stem/stromal cell
P	P value
PBS + BSA	Phosphate buffered saline + 0.5% bovine serum albumin
PE	Phycoerythrin

PPAR-γ	Peroxisome proliferator-activated receptor- γ
PVDF	Polyvinylidene fluoride
R1	Region/Gate 1
R2	Region/Gate 2
R3	Region/Gate 3
R4	Region/Gate 4
RBC	Red blood cell
RNA	Ribonucleic acid
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
S	The synthesis stage of the cell cycle
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SSC	Stromal stem cell
SSC	Side scatter
SSEA	Stage-specific embryonic antigen
STRO-1	Stromal cell factor-1
sub G0	Cells with less than one copy of DNA (dead/dying)
T3	T cell antigen receptor complex
TAMRA	Tetramethylrhodamine
Thy-1	Thymocyte-differentiation antigen-1
TLR4	Toll-like receptor 4
WBC	White blood cell

LIST OF SYMBOLS

α	anti
β	beta
°	degree
C	Celsius
cDNA	complementary DNA
cm	centimeter
ddCt	delta delta cycle threshold
e.g.	for example
et al.	and others
γ	gamma
g	gravitational force
≥	greater than or equal
≤	less than or equal
H+L	heavy plus light chain
IV	intravenous
kDa	kilo Dalton
kg	kilogram
L	liter
µ	mu chain
µg	microgram
µL	microliter
µm	micron
µM	micromolar
mg	milligram

mL	milliliter
mM	millimolar
Min/Max	minimum/maximum
mw	molecular weight
n	number
ng	nanogram
nM	nanomolar
#	number
%	percent
±	plus-minus
®	registered
SE	standard error
X	times

CHAPTER 1

GENERAL INTRODUCTION

Broad Objectives of Dissertation Research

Bone marrow is composed of many cell types. My research involved a particular subset of bone marrow cells known as mesenchymal progenitor cells (MPC). The MPCs of humans and other species have been studied for approximately forty years. Despite decades of MPC research, these cells are still relatively poorly understood compared to other well-defined bone marrow populations, such as hematopoietic stem cells (HSC). My overall objective was to gain a better understanding of what unique cell surface properties these putative MPCs possess. Long-term goals for my research are to identify candidate MPC surface markers to facilitate isolation of MPCs in future cell sorting studies and to evaluate the clinical usefulness of sorted MPCs in cell-grafting procedures. The horse was chosen as a model organism to supply large quantities of bone marrow cells from each aspirate, thereby facilitating multiple analyses of each sample.

The first objective of my research was to characterize the cell surface phenotype of putative equine MPCs derived from bone marrow. I was interested in how the cell surface markers changed over time in culture as the MPC population was established. My hypothesis was that the cell surface phenotype of bone marrow derived MPCs would be dynamic from cell isolation through one month culture. Results of my initial study demonstrated a number of changes in cell surface molecules during establishment of MPC cultures, confirming the hypothesis.

Contamination with adherent hematopoietic cells, such as monocytes and their descendants, creates a substantial obstacle to obtaining relatively pure MPC cultures for analysis. One way to reduce the level of hematopoietic cell contamination is to

sort bone marrow cells based on a cell surface protein that is differentially expressed between the populations. According to the accepted classification of human MPCs, the CD14 cell surface protein, also known as the lipopolysaccharide receptor (LPS-R), should be differentially expressed between MPCs and adherent hematopoietic cells. This protein should be present on the cell surface of monocytes, macrophages, and dendritic cells. According to the literature, it should not be detectable on the surface of MPCs. Therefore, my second hypothesis was that MPC colony formation would be enriched in the CD14 negative fraction of adherent, cultured bone marrow cells when separated from the CD14 positive hematopoietic fraction. Results of the second study did not confirm the hypothesis. In fact, the data indicated just the opposite; MPC colony formation was enriched in the CD14 positive fraction.

In the horse, my data suggested that putative MPCs came from a CD14 positive cell population. Based on current knowledge, that implies equine MPCs are descendants of a hematopoietic cell, or MPCs have previously been misclassified. This finding has important implications in the clinical application of MPCs in cell grafting procedures. If the goal for cell-based therapies is stromal tissue regeneration, putative MPCs do not appear to be a cell type capable of achieving this goal *in vivo*, despite their behavior during *in vitro* cell differentiation studies. If the goal for clinical application putative MPCs is to improve tissue healing, without the absolute need for "tissue regeneration", they may play an important role in cell-based therapies. The current literature suggests the latter goal is attainable, realistic, and a useful indication for MPCs clinically.

The following chapters expand upon the information contained in each of the above paragraphs. The remainder of this chapter focuses on important background information related to the field of MPC biology and bone marrow analysis. The second chapter describes the dynamic changes early in culture of bone marrow

aspirate during the establishment of the MPC population. The focus of the third chapter is the enrichment of MPC colony formation using the cell surface molecule CD14 to separate hematopoietic and putative stromal cell lineages. The final chapter summarizes the implications for clinical application of putative MPCs in cell grafting procedures.

History of Bone Marrow Research

Bone marrow cytological analysis was an innovative technique developed over 150 years ago to improve our understanding of the highly complex bone marrow tissue compartment. Morphology of bone marrow cells was first described by E. Neumann in 1868 [1]. Later, he described the transformation of "fatty marrow" into functional, red hematopoietic tissue in conditions of severe anemia. In the early twentieth century, Alexander A. Maximow was credited for confirming the Unitarian Theory of Hematopoiesis: that all blood cells arise from a common precursor [2]. Combined, these observations led to the important advances in the field of bone marrow evaluation, including the diagnosis and treatment of neoplasias such as leukemia, aplastic anemia from radiation sickness, and in infections such as tuberculosis [3].

Over the history of bone marrow research, techniques were developed to harvest samples from living patients in order to acquire bone marrow cells for cytological analyses. Pianese first attempted to obtain a diagnostic sample of bone marrow in 1903 via trephination of the femoral epiphysis to study hematologic conditions [1]. The first description of a sternal bone marrow aspirate was by C. Seyfarth in 1922 using a "puncture needle" [1]. In 1971, a new type of trephine needle was patented by Khosrow Jamshidi [4] which allowed for bone marrow tissue to enter the lumen of the needle without being crushed. Presently, there are many

different types of commercially available bone marrow aspirate needles; however most incorporate the cannula and stylet design developed by Jamshidi.

In the 1950's through 1970's, a team led by Edward Donnall Thomas at the Fred Hutchinson Cancer Research Center, pioneered work in the field of hematopoietic stem cell transplantation following bone marrow ablation [5, 6]. In 1990, Thomas received a Nobel Prize for his work on graft versus host disease in bone marrow transplantation. The first successful human bone marrow transplantation was performed by Robert A. Good in 1968 at the University of Minnesota to treat a boy with a severe sex-linked immunodeficiency [7]**.** Worldwide, about 30,000 autologous (donor is the recipient) and 15,000 allogenic (donor is a member of the same species, but is not the recipient) bone marrow transplants are performed annually in the treatment of a variety of diseases [8].

Bone marrow aspirate can be obtained from several anatomic sites. The most common site for bone marrow aspiration in people is the posterior iliac crest of the pelvis. This site provides a rich source of red marrow. Other less frequently utilized sites for aspiration of bone marrow in humans include the anterior iliac crest, sternabrae, proximal humerus, and the anteromedial surface of the tibia in young children. The anterior iliac crest is generally used only in patients who are obese, since their large body size makes the posterior iliac crest inaccessible. The sternabrae carries a small risk of inadvertent lung or cardiac puncture, leading to safety concerns, and therefore, less frequent usage in people. Finally, the marrow space in the proximal humerus and anteromedial tibia fill with yellow marrow with age, making them unsuitable donor sites in the adult.

Several anatomic sites are also used in horses for collection of bone marrow aspirate. The sternabrae are commonly used for bone marrow aspiration in the standing, sedated horse. Another frequent site utilized in the horse is the tuber coxae

of the pelvis. In addition to bone marrow aspiration, both of these sites are also important locations for acquisition of cancellous bone graft. A rarely reported site which has been used for bone marrow aspiration in young horses is the tibia [9]. The humerus has not been reported as an aspiration site in horses, although the region has been utilized in other species [10-13].

History of Mesenchymal Progenitor Research

In the 1970s, A.J. Friedenstein published a series of papers on the clonal nature of bone marrow stromal cells which initiated the field of bone marrow-derived mesenchymal progenitor cell (MPC) research [14, 15]. In 1999, M.F. Pittenger used flow cytometry to characterize the cell surface proteins expressed on cultured MPCs. He also demonstrated the capacity of MPCs *in vitro* to differentiate into cells of osteogenic, chondrogenic, and adipose lineages [16]. Since that time, well over 10,000 articles related to MPCs have been published. In spite of all of the research and interest in MPCs, there is still a great deal of controversy on the exact definition, characteristics, and therapeutic uses of MPCs. Basic guidelines have been established by the International Society for Cellular Therapy (ISCT) for the minimal criteria to define human Multipotent Mesenchymal Stromal Cells (MSC), including clarification of nomenclature [17]. According to the ISCT, the plastic-adherent cells isolated from bone marrow and other sources do not meet the generally accepted criteria for stem cell function, rendering the terminology mesenchymal stem cell inaccurate and potentially misleading to the public. Further, since MSC can be interpreted as either mesenchymal stem cell or mesenchymal stromal cell if not clearly defined, herein the more general term, mesenchymal progenitor cell (MPC) will be used when referring to this cell type. Besides adherence to tissue culture-plastic, according to the ISCT definition, humans MPCs must express the cluster of differentiation (CD) molecules

CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR molecules on their cell surface [18].

The cluster of differentiation (CD) molecule nomenclature classifies monoclonal antibodies by the epitope they recognize on the cell surface [19, 20]. If two or more specific monoclonal antibodies bind to the molecule of interest, a CD designation is assigned. If only one monoclonal antibody binds, a provisional indicator, "w" is included in the name. To date, more than 350 designations have been classified for humans. Although the CD classification system was initially developed for leukocytes, a number of other cell types, including stromal cells, have been phenotyped based on the presence or absence of CD molecules [21]. The classification of human MPCs using the CD phenotype has attempted to standardize the definition of MPCs using more objective criteria than simple plastic adherence.

In addition to cell surface properties, ISCT guidelines indicate that human MPCs must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [18]. Criteria used to define *in vitro* differentiation vary by desired tissue and include histochemical staining results, gene expression data, and chemical properties. For osteogenic differentiation, intracellular calcium/protein ratios can be used to demonstrate calcium accumulation in treated cells. Alkaline phosphatase (ALP) and alizarin red dyes are commonly used to stain for bone (ALP is a by-product of osteoblastic activity) and mineral (alizarin red stains calcium) deposition. Increased levels of expression of genes such as osteonectin and osteocalcin can also be used to provide evidence of osteogenic differentiation. For adipogenic induction, histological staining with Sudan black or Oil Red O is frequently used to assess lipid accumulation. Antibody staining for leptin, a hormone produced by adipocytes, can also be used to confirm adipocytes are functional [22]. Increased expression levels of genes such as peroxisome proliferator-activated receptor- γ (*PPAR-γ*) and fatty acid

binding protein 4 (*FABP4*) also provide supporting evidence of adipogenesis. Chondrogenesis can be evaluated via immunohistochemical staining by comparing levels of collagen staining (e.g. collagen type II staining provides supporting evidence of chondrogenesis, type X suggests hypertrophic cartilage, and other collagens such as types I and III suggest fibrous tissue formation). Other stains such as Safranin O/fast green and Toluidine blue can be used to assess matrix metachromasia, which is indicative of glycosaminoglycan synthesis. Gene expression levels of the various collagen types and matrix components such as aggrecan provide additional support of chondrogenesis.

Putative MPCs have been isolated from a variety of human tissue sources including bone marrow, umbilical cord blood, adipose tissue, peripheral blood, muscle, periodontal tissue and periosteum. Bone marrow has been the most studied and utilized source of MPCs to date in people. Therefore, my research has focused primarily on equine MPCs acquired from bone marrow aspirates.

Anatomy of Bone Marrow

Bone marrow is found in the medullary cavities of bones and contains a heterogeneous mixture of cells including hematopoietic cells and their precursors, adipocytes, endothelial cells, fibroblasts, and bone cells such as osteoblasts and osteoclasts. Bone marrow can be classified as either yellow (containing primarily adipocytes) or red (containing mainly hematopoietic cells). Neonatal human bone marrow is exclusively red marrow and remains as such for about seven years. With aging, bone marrow becomes more of the yellow form unless a stimulatory event, such as severe blood loss, causes it to convert back to red marrow. In adults, red marrow is found primarily in the end of long bones, near the hip, in the sternabrae, vertebrae, ribs and skull. Red and yellow bone marrow spaces are found in similar anatomic

locations in the horse. Rich sources of red marrow in the horse include the sternabrae as shown in **Figure 1.1**.

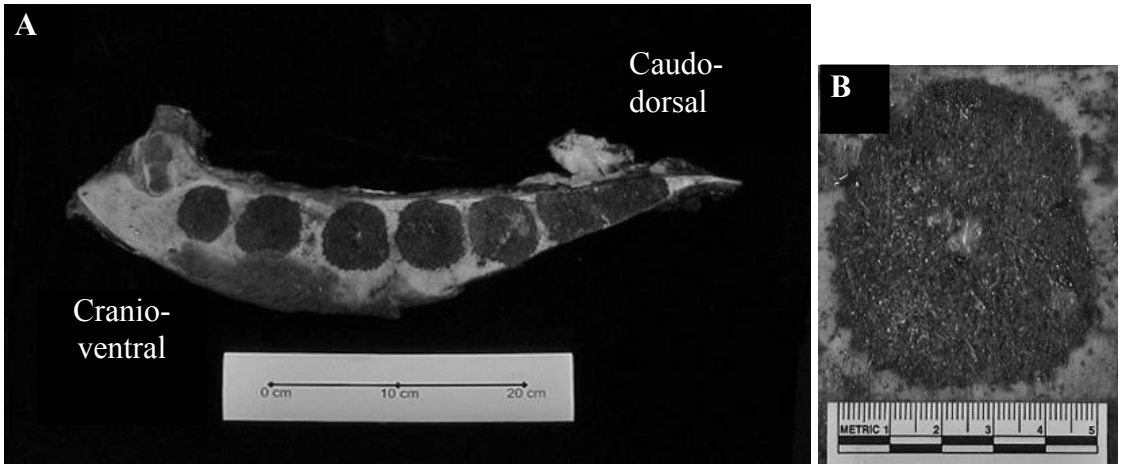

Figure 1.1 Cross section of an equine sternum (A) revealing seven large marrow cavities that can be accessed to obtain bone marrow aspirate. An enlarged photograph of the third marrow element (B) further depicts the rich reservoir of bone marrow.

Red bone marrow is highly vascular and contains fibrous networks of cells, blood vessels, bone, and fibrous tissue. The stroma provides a microenvironment, or niche, to facilitate and regulate the parenchymal cells which carry out hematopoiesis. Hematopoietic stem cells give rise to leukocytes (white blood cells), erythrocytes (red blood cells), and thrombocytes (platelets). Immature blood cells are normally blocked from leaving the marrow because they lack cell surface adhesion proteins necessary for diapedesis. Other stem cells found in the marrow include endothelial stem cells which give rise to blood vessels and MPCs which give rise to cells such as adipocytes, chondroblasts, and osteoblasts *in vitro*.

The Horse as a Model Organism

Expanded knowledge of the behavior of MPCs in nonhuman biomedical research animal models both *in vitro* and *in vivo* is an important step for future application of these cells in human or animal patients. The horse is a good candidate for MPC research due to its size (which allows for relatively large sample collections). Further, horses are a well developed model system to test therapeutic applications in cartilage, tendon, and bone healing, facilitating the application of MPC preparations to *in vivo* testing. The horse has a distinct advantage as a model organism for MPC studies as multiple tissues can be sampled using minimally invasive techniques, under light sedation and local anesthesia. Autologous grafting procedures are possible because MPCs can be collected non-lethally from the same horse, eliminating the potential confounding factors of transplanting allografts into immunocompetent recipients. Putative MPCs have been isolated from a variety of equine tissues including bone marrow, umbilical cord blood, peripheral blood, adipose tissue, and muscle [23-29].

The horse has been a long standing model for osteoarthritis research. The first study examining arthritis in the horse was reported over 50 years ago [30]. The equine model is well suited for application to human joint disease because of the horse's athletic nature, and the large biomechanical forces which equine joints must withstand. In contrast, smaller mammals, such as mice or rabbits, cannot sustain biomechanical forces remotely comparable to those measured in human joints. A publication utilizing horse MPCs with measurements of *in vivo* cartilage repair has become available [31]. Numerous additional studies measuring chondrogenic potential of equine MPCs *in vitro* have been reported [25, 32-37].

Horses are also important models for the study of tendon healing. Studies range from the effect of extracorporeal shock wave therapy [38], microcurrent tissue

stimulation *in vitro* [39], to measurement of growth factors and tendon matrix components in normal and injured tendons [40-44]. The induction of equine superficial digital flexor tendonitis using collagenase injection [40-43, 45, 46] is a well-established model which is frequently utilized in the study of MPC application to tendon defects. Since horse tendons experience forces that meet or exceed the magnitudes of force experienced during healing of human tendon injuries, the horse is a suitable model system for extrapolation to human tendon lesions.

A model system to evaluate bone healing has been well established for the horse. The metatarsal osteotomy procedure is a minimally invasive way to measure bone stability and gap healing [47, 48]. Interestingly, osteogenic cell populations have been found to vary at different skeletal sites in the horse [49], emphasizing the need to utilize a highly standardized model system.

The horse has a number of advantages that make it well suited as a model organism for bone marrow research; however some disadvantages are present. Many of the antibody reagents used to isolate human MPCs do not recognize the equine form of the respective molecules, leading to challenges in antibody-based isolation and analysis of putative equine MPCs. Further, many of the advanced transgenic model systems such as green fluorescent protein (GFP) transgenesis, which can be used to track GFP donor cells in non-GFP recipients, cannot be applied to horses.

Current Therapeutic Applications of Mesenchymal Progenitor Cells to Humans

Putative mesenchymal progenitor cells have been used in a number of studies for treatment of a variety of disease conditions. Most human studies are still in Phase 1 clinical trials to demonstrate safety in MPC cell-grafting procedures. While a rapidly growing quantity of clinical data supports the safety of mesenchymal stem cell transplants, the efficacy data are variable and of mixed benefit [50]. Recent clinical

trials tested MPC treatment for a variety of indications including amyotrophic lateral sclerosis [51], radiation-induced lung injuries [52], and augmentation to hematopoietic stem cell transplants [53]. Unfortunately, these studies do not show a significant treatment effect outside of possible immune modulation and anti-inflammatory properties. When bone marrow derived MPCs have been studied for heart disease [54], stroke [55], and other neurodegenerative disorders, progress has also been mixed and without significant benefit [50]. To date, only one prospective study of human MPC transplantation into a cartilage defect has been published [56]. This study found no significant clinical improvement in patients who received MPCs compared to their cell-free controls, with minor improvements in biopsy histologic scores. One area MPCs have been utilized with apparent signs of positive therapeutic effect is the control of graft-versus host disease [57], supporting the concept that MPCs function primarily through immune modulation.

The International Society for Stem Cell Research (ISSCR) has created a series of guidelines for responsible translational stem cell research that highlight some of the challenges occurring in the clinical application of human MPCs [58]. Specifically, ISSCR wants to prevent exploitation of patients' hopes by preventing "stem cell therapies" that lack credible scientific rationale, transparency, oversight, or patient protections. The ISSCR recommends rigorous preclinical testing in animal models whenever possible because it is difficult to predict *in vivo* MPC behavior from *in vitro* studies alone. They also call on researchers to publish positive results, negative results, and adverse events to promote transparency in clinical therapeutic trials. My research adds knowledge to the field of MPC biology using a well accepted model organism with the potential for translational impact on a variety of human MPC clinical applications.

Questions to be Addressed by Dissertation Research

Bone marrow aspirate contains a heterogeneous mixture of hematopoietic and non-hematopoietic cell types that become more homogeneous over the first three weeks of culture. I wanted to develop a better understanding of the temporal changes equine bone marrow cells undergo during early culture expansion. My hypothesis was that the cell surface phenotype of bone marrow derived MPCs would be dynamic from cell isolation through one month culture. There are no uniformly accepted definitive phenotypes or surface markers that can be used to identify or isolate MPCs from native bone marrow aspirate samples [59]. In fresh bone marrow aspirate, cells of varying maturity in both hematopoietic and non-hematopoietic lineages are present, with varying levels of surface protein expression within each population, making separation of cells from distinct lineages difficult. During early culture, the proportion of hematopoietic cells committed to terminal differentiation is reduced via spontaneous apoptosis and removal due to non-adherence, leading to a more uniform population of mesenchymal cells. Most studies evaluate MPC cell surface markers after expansion in culture in order to obtain sufficient cell numbers for analysis [60-63]. However, there are conflicting reports of MPC marker protein expression patterns when comparing phenotypes of freshly sorted MPCs to expanded MPCs [64, 65]. These studies suggest that the phenotype of MPCs is dynamic during isolation and culture processes.

The broad aim of the studies in this thesis was to identify some of these early changes in cell-surface phenotype. Long term, these changes might be capitalized on for the purpose of improved cell separation. Chapter 2 describes my preliminary work in defining the molecular characteristics during establishment of putative MPC cultures. Chapter 2 also discusses the importance of temporal gene expression analysis as a component of the characterization of putative MPCs. I sought to evaluate

dual protein/gene analysis to assess the usefulness of gene expression data in confirming negative protein results and accounting for kinetic changes of transcription and translation in bone marrow cells during expansion in culture. Gene expression data has been utilized primarily in previous MPC studies to evaluate differentiation capacity into terminally differentiated tissues (e.g. collagen type II for cartilage; osteonectin for bone) [16, 66]. When monoclonal antibodies are used to immunophenotype cells in uncharacterized tissue, gene expression data provides supporting evidence for protein expression in the cells and helps to validate the reactivity of the antibody.

An important step in the long-term goal of improved cell separation is identification of molecules that are differentially expressed between MPCs and hematopoietic cells. MPCs of humans and other species are reported to lack expression of the cluster of differentiation (CD) marker CD14, also known as the lipopolysaccharide receptor (LPS-R) on their cell surface [18, 67]. Chapter 3 describes the analysis of CD14 expression in equine MPCs. I aimed to evaluate CD14 expression patterns in equine bone marrow as a potential negative MPC marker. My hypothesis was that MPC colony formation would be enriched in the CD14 negative fraction of adherent, cultured bone marrow cells when separated from the CD14 positive hematopoietic fraction. Since the CD14 cell surface protein is typically found on monocytes and closely related hematopoietic cells, it appeared to be a good candidate protein for differential expression between adherent hematopoietic cells and putative MPCs. There are no reports of CD14 gene or protein expression status in equine MPCs. As a preliminary step in this study, I utilized temporal evaluation of gene and protein expression to assess the levels of CD14 expression in freshly isolated cells through one month of culture. In fresh marrow, cells of early hematopoietic origin did not express CD14 or other hematopoietic specific markers such as

CD11a/CD18 uniformly in the expected populations, complicating separation of hematopoietic versus non-hematopoietic cells. It was proposed that many freshly isolated bone marrow cells of hematopoietic origin were too immature to express specific surface proteins that would allow for distinction between cell lineages. Based on preliminary studies, I determined that short term culture (approximately 2 days) allowed adherent cells in culture to reach a stage when they could express CD14 molecule if that was their lineage commitment (hematopoietic) and be separated with relative purity from cells that did not express CD14 (non-hematopoietic). Utilizing a technique called magnetic activated cell sorting (MACS), I expected that lineage committed monocytes and neutrophils (which are positive for CD14 expression) could be separated from putative MPCs (which are reported to be CD14 negative in other species) in bone marrow cultured 2 days using a mouse anti-equine CD14 antibody. In contrast to my expected outcome, the anti-equine CD14 antibody concentrated the equine MPC colony forming units (CFU) in the CD14 positively selected cells, while the CD14 negative fraction of the bone marrow was nearly devoid of CFU, rejecting my hypothesis.

Given the surprising nature of my results, I wanted to press further in the study of CD14 expression in MPCs by examining putative MPCs for long term retention of CD14 expression. Over time, I observed a dramatic decrease in CD14 expression by the cultured bone marrow cells; however, low levels of expression were detected throughout culture. These results suggest that cells with initial high levels of CD14 (CD14 positive on MACS separation) are either differentiating into another cell type or down-regulating their expression in response to the culture environment.

In order to determine if the low level of CD14 detection in MPCs was real or anomalous, I proposed to stimulate MPCs with lipopolysaccharide (LPS) to see if CD14 expression in MPCs would be up-regulated in response. Established cultures

of putative MPCs were tested and found to be LPS responsive, with an increase in CD14 gene and protein expression following LPS exposure. These results suggest CD14 cell surface receptors are present and poised for upregulation in MPCs.

Most characterization studies of culture expanded MPCs in humans and other species include use of a proteolytic enzyme called trypsin as part of their cell harvest protocols to detach adherent cells prior to flow cytometry analysis. I aimed to determine if damage to the CD14 epitope by trypsin could offer a possible explaination for the descrepency between previous MPC characterization studies and my findings. I wanted to test the sensitivity of the equine CD14 surface protein receptor to trypsin under typical cell dissociation conditions. Established cultures of MPCs were harvested using either 0.25% trypsin or Accumax® (Innovative Cell Technologies Inc. San Diego, CA) cell dissociation solutions. Flow cytometry results showed a significant drop in mean fluorescence intensity detection when trypsin was used for cell harvest compared to cells harvested with Accumax®. In fact, when trypsin was used, levels of CD14 were undetectable and had mean fluorescence intensity comparable to the negative control antibodies. Equine CD14 is clearly trypsin labile, invalidating flow cytometry data collected following trypsinization. This could be a factor in flow cytometry analyses in other species and could be a plausible explanation for the discrepancy between my detection of CD14 protein in equine MPCs and the negative CD14 expression results reported in other species.

Only certain lineages of hematopoietic cells (e.g. monocytes, macrophages, dendritic cells, and to a lesser extent neutrophils), are known to express CD14. By definition, MPCs of other species are classified as non-hematopoietic in lineage because they lack expression of specific hematopoietic molecules such as CD14. The expression of CD14 by equine MPCs supports the concept that these cells arise from a hematopoietic lineage precursor and may not be truly “stromal” in origin (if a cell

must be CD14 negative to be stromal). Based on my results, I believe that equine MPCs are likely differentiated descendants of a monocytic precursor cell that have down regulated their expression of CD14 in response to the culture environment. It is also possible that previous descriptions of MPCs have misclassified these stromal cells as CD14 negative, when they should have been classified as low positive for CD14 expression. Further studies are needed to elucidate which of the two possibilities is true, as cellular lineage has implications for the application of these cells clinically. If MPCs are descendants of hematopoietic cells, can they be expected to form a differentiated stromal tissue such as cartilage or muscle *in vivo*? What is their therapeutic value and what should be the realistic expectations for outcome when evaluating their clinical benefits? If they are a misclassified stromal cell, can CD14 be used as a positive selection marker early in culture for enriched isolation of MPCs and improve the quality and speed for cell graft preparation? In future studies I hope to address these questions and expand current knowledge of the equine MPC. The field of MPC biology holds great potential to have a significant impact on a host of human and animal diseases, but steps need to be taken to ensure transparency and credible scientific rationale in their clinical use.

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CHAPTER 2

TEMPORAL ANALYSIS OF EQUINE BONE MARROW ASPIRATE DURING ESTABLISHMENT OF PUTATIVE MESENCHYMAL PROGENITOR CELL POPULATIONS

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Abstract

Mesenchymal progenitor cells (MPCs) are often characterized using surface markers after expansion and treatment in culture. There are no studies directly comparing gene and protein markers in undifferentiated samples during the very early phases of culture. The goal of this study was to evaluate temporal gene and protein expression changes during establishment of equine MPC cultures. Bone marrow aspirate was obtained from thirty five horses and processed by density gradient centrifugation. In freshly isolated bone marrow, mononuclear cells had variable expression of CD44, CD11a/CD18, CD90 and CD45RB cell surface molecules. After two hours of culture, bone marrow mononuclear cells had a phenotype of $CD44^{hi}$, $CD29^{hi}$, $CD90^{lo}$, $CD11a/CD18^{hi}$ and $CD45RB^{lo}$. Isolated mononuclear cells were analyzed by flow cytometry and RT-qPCR at 2, 7, 14, 21 and 30 days of culture. At all culture time points, gene expression was in agreement with cell surface protein expression. In established cultures of MPCs, cells remained robustly positive for CD44 and CD29. The proportion of positive cells and the mean fluorescence intensity of positive cells increased for CD90 expression as MPC cultures became more homogeneous. Inversely, the population of cells in culture decreased expression of CD11a/CD18 and CD45RB molecules over time. The decreased expression of the latter molecules makes these useful negative markers of established MPC cultures under normal expansion conditions. The results of this study demonstrate numerous dynamic changes in cell surface molecule expression during early establishment of MPC populations which may aid to improve MPC isolation methods for research or therapeutic applications.

Introduction

Mesenchymal progenitor cells (MPC) have been studied extensively in many species since the first report by Friedenstein over thirty years ago [1]. Characterization studies of established human MPC cultures using differentiation assays, gene expression analysis, and cell surface protein markers have been performed for nearly a decade [2]. Most studies evaluate MPC cell surface markers and gene expression after expansion in culture in order to obtain sufficient cell numbers for analysis [3-6]. However, there are reports of conflicting results in MPC marker protein expression patterns when comparing phenotypes of freshly sorted MPCs to expanded MPCs [7, 8]. These studies suggest that the phenotype of MPCs is dynamic during isolation and culture processes.

Temporal changes in cell surface protein expression during expansion in culture have been reported in only a few studies. In the original MPC description by Pittenger et al. [2], population enrichment from day two through fourteen was described based on flow cytometric measurement of SH2 and SH3 expression, but full cell surface protein characterization was not reported until passage one or two using expanded cells. Another study reported no temporal changes in cell surface phenotype for bone marrow cells after they had reached confluence in culture compared to their next five passages [9]. Although these studies have added important information concerning cell expansion, early immunophenotype changes remain incompletely understood.

The use of gene expression data in most MPC studies has focused primarily on assessment of MPC differentiation capacity into terminally differentiated tissues (i.e. collagen Type II for cartilage; osteonectin for bone) [2, 10]. When monoclonal antibodies are used to immunophenotype cells in a previously uncharacterized tissue type, gene expression data provides supporting evidence for protein expression in the

tissue and helps to validate the reactivity of the antibody. The advantage of dual protein/gene analysis is to confirm negative protein results and account for kinetic changes of transcription and translation.

Early bone marrow cultures contain a heterogeneous mixture of cell types, which become more homogeneous over the first three weeks of culture. There are no uniformly accepted definitive single or combination of cell surface markers for isolation of MPCs from uncultured samples [11]. In fresh bone marrow aspirate, cells of varying maturity in both hematopoietic and non-hematopoietic lineages are present, with varying levels of surface protein expression within each population, making separation of cells from distinct lineages difficult. During early culture, the proportion of hematopoietic cells committed to terminal differentiation is reduced via spontaneous apoptosis and removal due to non-adherence, leading to a more uniform population of mesenchymal cells. In the present study, my hypothesis was that the immunophenotype of bone marrow cells was changed during the very early phases of MPC culture establishment as the cell population became more homogeneous. The goal of this study was to evaluate both gene and protein expression of cell surface markers to characterize MPCs using flow cytometry and RT quantitative PCR (RT-qPCR) throughout culture duration. The results of this study may aid to improve MPC selection and isolation methods for research or therapeutic uses.

Materials and Methods

Study design: Candidate antibodies were tested for reactivity and specificity with equine cell surface antigens. Subsequently, cell surface molecules of uncultured bone marrow cells were analyzed using flow cytometry. Bone marrow cells were cultured and harvested on 2, 7, 14, 21, and 30 days for analysis of cell surface proteins and

gene expression. All procedures were performed in compliance with institutional guidelines for research on animals.

Antibody validation: To validate reactivity of antibodies with equine cells, peripheral blood cells were used as positive and negative controls. Whole blood (30 mL) was collected from five horses for antibody validation. Blood samples were drawn into preservative free heparin to a final concentration of 33 units/mL. Candidate equine and human monoclonal antibodies tested are listed in **Table 2.1**. Whole venous blood was processed prior to flow cytometry analysis using density gradient centrifugation to remove the majority of red blood cells as previously described [12].

Validation of antibody specificity: The CD44 and CD11a/CD18 antibodies have been previously validated as specific for their respective molecules in the horse ([13, 14]. The CD11a/CD18 antibody (clone CZ3.2) identifies a non-covalently linked heterodimer consisting of a 180kDa α chain (CD11a) and a 95kDa β chain (CD18) using immunoprecipitation under reducing conditions[13, 14]. The CD44 antibody (clone CVS 18) identifies a heavily glycosylated molecule of 65-100kDa [13, 14]. On a 12% SDS-PAGE analysis, a "smear" was produced approximately in the 100kDa position, indicating that the precipitated molecule was heavily glycosylated. The analysis was repeated after endoglycosidase F treatment of the precipitate, and a single 76kDa band was produced in both reducing and non-reducing conditions [13, 14]. The CD44 antibody has also been shown to react with protein produced by a cDNA encoding equine CD44 molecule in a COS cell expression system [15].

For CD90, CD29, and CD45RB antibody validation analyses, whole cell lysates were prepared from fresh peripheral blood leukocytes and from red blood cells with platelets. Western blot analyses were performed to determine if the reactive candidate antibodies bound proteins of the expected size based on previous literature, protein size similarity to other species, or predicted equine sequences. The CD90

Table 2.1 Candidate antibodies tested to determine the changes in equine MPC cell surface antigens in uncultured samples and subsequent propagation of cells in culture. Alternate antigen names are listed in parentheses.

ANTIBODY TESTED	SPECIES ANTIBODY IS PRODUCED AGAINST (COMPANY, CATALOG #)	POSITIVE HUMAN CELLS	POSITIVE EQUINE CONTROLS TESTED	NEGATIVE EQUINE CONTROLS TESTED
CD29 (Integrin β1)	Human (Beckman Coulter, Fullerton, CA; cat # 6603177)	Lymphocytes, monocytes, granulocytes-low, platelets, fibroblasts, endothelial, NK cells, thymic /lymph node cells	Lymphocytes, monocytes, granulocytes, platelets	Red blood cells
CD44 (H-CAM)	Equine (Serotec, Raleigh, NC; cat # MCA 1082)	Hemato and non-hemato except platelets	Leukocytes	Red blood cells platelets
CD90 (Thy-1)	Canine (VMRD, VMRD Inc, Pullman, WA; cat # DH24A)	Hematopoietic stem cell subset, neurons, fibroblasts, stromal cells	Granulocytes	Lymphocytes
CD45RB (LCA)	Bovine (VMRD Inc, Pullman, WA; cat # DH16A)	Granulocyte/ lymphocyte subpopulations	Granulocytes, lymphocytes	Red blood cells, platelets
CD11a/CD18 (Integrin αL, LFA-1)	Equine (CZ 3.2 courtesy of Dr. D. Antczak, Cornell University; clone 116.2D11B10)	All leukocytes	Leukocytes	Red blood cells, platelets

Table 2.1 (Continued)

Stro-1	Human (R &D Systems, Minneapolis MN; cat # MAB1038)	Human stromal cell precursors	None available	Leukocytes
CD13 (APN)	Equine (Serotec, Raleigh, NC; cat # MCA 1084)	Granulocytes, monocytes and their precursors, endothelium, epithelium	Granulocytes, monocytes	Lymphocytes
SSEA 1	Mouse (DHSB University of Iowa; cat # MC-480)	Human ES cells	None available	Leukocytes
SSEA 3	Rat (DHSB University of Iowa; cat # MC-631)	Human ES cells	None available	Leukocytes
SSEA 4	Human (DHSB University of Iowa; cat # MC-813-70)	Human ES cells	None available	Leukocytes
CD34 (Mucosialin)	Human (BD Biosciences, San Jose, CA; cat # 555824 & cat # 340666)	Human hematopoietic stem and endothelial cells	None available	Mature leukocytes

Table 2.1 (Continued)

CD3 (T3)	Equine (courtesy of Myra Blanchard, UC Davis; clone UC F6G-3.3)	Mature T lymphocytes	T lymphocytes	Granulocytes, monocytes
MHC class I	Equine (CZ3 courtesy of Dr. D. Antczak, Cornell University; clone 117.1B12C11)	Leukocytes and almost all nucleated cells in the body	Leukocytes	Early allantochorion cells
MHC class II	Equine (CZ11 courtesy of Dr. D. Antczak, Cornell University; clone 130.8E8D9)	Mature lymphocytes	Lymphocytes	Granulocytes
Mouse α Parvo Virus	Canine (courtesy of Dr. D. Antczak, Cornell University; tissue culture supernatant)	Parvo virus	None available	All equine cells should label negatively

CD, cluster of differentiation

H-CAM, homing-associated cell adhesion molecule

Thy-1, Thymocyte-differentiation antigen-1

LCA, leukocyte common antigen

LFA-1, lymphocyte function-associated antigen-1

APN, Aminopeptidase N

SSEA, stage-specific embryonic antigen

T3, T cell antigen receptor complex

antibody was expected to detect an approximately 17kDa protein, similar in size to the equivalent human protein. Similarly, the CD29 antibody was expected to detect an approximately 130kDa protein based on the size of the human protein. The CD45RB antibody was expected to have one or more bands less than 150kDa based on the multiple isotypes of the human protein. To test the CD45RB and CD29 antibodies, proteins from cell lysates were resolved on 7.5% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels, which were subsequently transferred to polyvinylidene fluoride (PVDF) membranes and probed with the relevant antibody. A 15% SDS-PAGE gel was used to resolve cell lysates for subsequent analysis of the CD90 antibody following protein transfer to a PVDF membrane.

An immunoprecipitation was performed in addition to Western blot analysis for the CD29 antibody, using an unconjugated version of the antihuman CD29 (Beckman Coulter, clone 4B4LDC9LDH8) used in this study. A 7.5% SDS-PAGE gel was used to resolve the immunoprecipitated products. Following protein transfer, the PVDF membrane was probed with antibody known to recognize human β1 integrin (Calbiochem, clone 4B7-CP26).

Bone marrow aspirate collection and cell isolation: Bone marrow aspirate was used to assess changes in cell surface markers over time and for tri-lineage (cartilage, bone, and adipose) differentiation. Bone marrow aspirate was collected from the sternabrae of thirty five horses (11 males and 24 females, age range 6 months - 20 years) under standing sedation with xylazine hydrochloride (0.55 mg/kg IV) and local anesthesia using 2% lidocaine hydrochloride (10 mL/site). Samples were collected in preservative free heparin (American Pharmaceutical Partners Inc, Schaumburg, IL) to a final concentration of 33 units/mL.

The aspirate (60 mL) from each horse was diluted to 180 mL total volume using phosphate buffered saline + 0.5% bovine serum albumin. The white blood cell

fraction of the sample was enriched and the majority of red blood cells were removed by layering each 30 mL aliquot of dilute sample on Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) for density gradient centrifugation, as described for antibody validation. Samples were resuspended in 50 mL MPC culture media (see below) prior to cell counting using a hemocytometer. Approximately 2-9 x 10^8 bone marrow mononuclear cells (BMMNC) were obtained per sample using this method. A portion (~10 x 10^6 cells) of the uncultured bone marrow aspirate samples from all thirty five horses were analyzed using flow cytometry. In a subpopulation of horses (n=8), samples of bone marrow aspirate before and following density gradient centrifugation were submitted for cytological analysis.

Samples from some horses (n=14) were used only for antibody validation and were not cultured. The remaining samples (n=21) were subsequently cultured as described below. A fraction of the cultured bone marrow samples (n=6) were cultured for 14 days and then utilized for analysis of DNA content to determine the cell cycle state. Samples from additional horses (n=3) were cultured for twenty-one days and then subjected to tri-lineage differentiation assays. Some cultured samples (n=6) did not have sufficient cell numbers to complete analysis at all time points; yet they were used for flow cytometry at one or more culture time points to check for repeatability or alterations in cell surface protein expression. Sufficient cell numbers for protein and gene expression analysis at all time points of culture were available from the remaining horses (n=6).

MPC expansion in culture: BMMNCs were plated onto 10 cm diameter tissue culture plates at a density of approximately 300,000 cells/cm^2 (20 x 10^6 cells/plate). Cells were cultured at 37°C in a 5% CO_2, 95% air atmosphere at 5% humidity. Cells were cultured in media containing Dulbecco's Modified Eagle's Medium (DMEM, glucose at 1000 mg/L), 2mM L-glutamine, penicillin (100 units/mL), streptomycin

(100 units/mL), basic fibroblastic growth factor (bFGF, 1 ng/mL) and 10% fetal bovine serum. One-half of the media (5 mL) was removed at 24 hours of culture and replaced with fresh media. Subsequently, media were exchanged every 72-96 hours. At sub-confluence of 70-90%, cells were passaged 1:3 using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, San Diego, CA) and plated at a density of about 10,000 cells/cm^2. Approximately 10 x 10^6 cells from each sample was analyzed by flow cytometry for cell surface protein expression at two hours and on days 2, 5, 7, 14, 21 and 30 of culture. Cells were analyzed at these time points to evaluate the changes in cell surface proteins over time, and to characterize the cells prior to performing differentiation assays.

Flow cytometry analysis: Cell surface markers of putative stemness were assessed using flow cytometry. Cells were pelleted in aliquots containing 1 x 10^6 cells and labeled for cell surface molecules selected from a panel of monoclonal antibodies known to define human MPCs (**Table 2.1**). Cells were treated with a twenty minute blocking step using 10% normal goat serum in Fluorescent Activated Cell Sorting Buffer (FACS-Buffer; phosphate buffered saline containing 2.5% fetal bovine serum). The cells were pelleted, washed with FACS-Buffer and pelleted again. Cell pellets were resuspended in fluorescent-conjugated or unconjugated primary monoclonal antibody and incubated for 45 minutes at 4°C. Cells were then washed, a second fluorescent-conjugated goat anti-mouse Immunoglobulin G (IgG) or Immunoglobulin M (IgM) antibody (Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE)-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) or IgM μ Chain Specific, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) was applied to the unconjugated antibodies, and the samples were incubated for an additional 45 minutes at 4°C. The CD29 antibody was directly conjugated with PE (read at FL2); all others were labeled with FITC-conjugated secondary antibody (read at FL1). Cells were resuspended in

FACS-Buffer and analyzed on a FACSCaliber (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with a 488μm argon laser and BD Cell Quest™ analysis software (BD Biosciences, San Jose, CA). Cells not treated with antibody, and cells exposed to mouse anti-parvo-virus antibody and FITC or PE-conjugated secondary antibodies were used as negative controls. The settings for the flow cytometric analyses determined less than 2% positive cells for the control antibodies. Data were collected on $1x10^5$ cells for each sample regardless of size and granularity to prevent bias in gating.

For culture expanded cells, flow cytometry analysis was performed on days 2, 7, 14, 21, and 30 following isolation. Supernatant was removed and adherent cells were lifted from the plate using Accumax® solution (1 mL/15cm^2) to prevent damage to cell surface proteins and avoid cellular clumping. Cells were processed and analyzed by flow cytometry as described above, except dot plot settings were adjusted to a logarithmic scale in the cultured cells to include large, granular cells. Flow cytometric analysis of cell surface molecule expression was performed in the gate determined to contain dividing cells based on the results from the propidium iodide DNA staining assay described below. Data for mean fluorescence intensity for the pertinent (reactive) antibodies was collected and compared between time points. The mean fluorescence intensities of all cells (not just positive) in the relevant gate were recorded.

Propidium iodide DNA staining assay for cell cycle analysis: Propidium iodide can be used to determine DNA content in cells and identify populations of cells undergoing division. A reported feature of MPCs is their ability to proliferate [2]. To determine the region of cell division, samples (0.5 x 10^6 cells) from six cultures were collected on day 14 and resuspended in 500 μL hypotonic propidium iodide solution containing 0.05 mg/mL propidium iodide, 1 mg/L sodium citrate and 0.1% triton x-

100 [16]. Samples were protected from light and incubated at 4°C until analysis. Samples were analyzed by flow cytometry on FL2. Histograms were plotted for each cell population on a linear scale. The DNA content is proportional to the mean fluorescence intensity, and consequently indicates the stage of cell division (G0/G1, S, G2, M; cells in sub G0 are dead) [17].

RNA extraction and One-Step Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR): Gene expression analysis was included to confirm negative protein results and account for kinetic changes in transcription and translation. At the same time points when cells were analyzed by flow cytometry, RNA was extracted from approximately 1-3 x 10^6 cells of the corresponding samples using Trizol® (Life Technologies, Invitrogen, Carlsbad, CA) according to the manufacturer's directions. RT-qPCR was performed to provide supporting evidence that gene expression levels were consistent with cell surface protein expression levels. RNA quantity and quality were determined using a Nanodrop® spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE), and visualization of 18 and 28S ribosomal subunit bands on 0.8% agarose gels. Gene segments were cloned and novel sequence data files were submitted to Genbank (accession numbers, EF442070 for *CD13*; EF442071 for *CD29*; EF576851 for *CD45*; EU881920 for *CD90*; and EU881921 for *CD11a*). A portion of the *CD44* gene was also cloned and agreed with previously reported data (X66862).

Total RNA was reverse transcribed and amplified using the One-Step RT-PCR technique and the ABI PRISM 7900 HT Sequence Detection System **(**Applied Biosystems, Foster City, CA). The primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software Version 2.0b8a (Applied Biosystems, Foster City, CA). All probes and primers were designed using equine specific sequences

published in Genbank, or sequenced in our laboratory (**Table 2.2)**. Since several isoforms (five in humans) of *CD45* exist, primers and probes were designed to detect as many equine isoforms as possible (equivalent to four of the five human isotypes). Two genes (*CD45* and *CD11a*) did not reach a C_T value in later time point samples. Therefore, normalized copy numbers/ng of RNA values for each gene were calculated. A quantity value of 1 was assigned to samples which did not reach a C_T.

MPC differentiation assays: To verify that cultured bone marrow cells were capable of tri-lineage differentiation, 10 x 10^6 culture-expanded cells from three horses were used for adipogenic, osteogenic, and chondrogenic induction assays.

Adipogenic induction: Aliquots of MPCs (0.2 x 10^4 cells /well) were treated with 5% rabbit serum (lot 24129, Innovative Research, Novi, MI) in culture media to induce adipogenesis [18].

Media were changed at day 4 following induction. Samples were collected on days 1, 3, and 7 post induction. To assess adipogenic differentiation, cells were fixed in 4% paraformaldehyde, incubated in a solution containing oil-red-O for 10 minutes to stain for lipid inclusions, and counterstained with hematoxylin. Stained samples were imaged using standard microscopy and graded positive or negative for oil-red-O staining.

Osteogenic induction: Aliquots of MPC (0.2 x 10^4 cells /well) were treated with 100nM dexamethasone, 10mM β-glycerophosphate and 50μM ascorbic acid (all Sigma-Aldrich, Inc. St. Louis, MO) in low glucose DMEM / 10% fetal bovine serum media. Media were changed at day 4 for the 7 day culture samples. Samples were collected on days 1, 3 and 7 following induction to assess early osteogenic differentiation. To assess calcium accumulation, cells were fixed in 4% paraformaldehyde and incubated in 2% aqueous alizarin red S (Sigma) for 3 minutes,

Table 2.2 Primers and probes utilized in RT q-PCR of MPC marker genes. The 18S ribosomal subunit was used to normalize gene expression.

Gene	Forward Primer	Reverse Primer	Probe
18S	5'CGGCTTTGGTGAC TCTAGATAACC-3'	5'-CCATGGTAGGC ACAGCGACTA-3'	5'-TCGAACGTCTGCCCTA TCAACTTTCGAT-3'
CD44	5'-TCCACCCCAA CTCCATCTGT-3'	5'-TGAAGCAATAGGT GTCGTACTGAGA-3'	5'-CCGCCAACAA CACGGGCG-3'
CD29	5'-GACAAGGTGAGCAA TAGAAGGATAATC-3'	5'-TTGGTGGCATTG TTTTACCAAA-3'	5'-CACATCATTTTCCAAG TGACACTGTCCATCA-3'
CD90	5'-CCGTGAGACAAA GAAGCATGTG-3'	5'-CCTGATGTTGTAC TTGCTGGTGAAG-3'	5'-TGAGCACACATAC CGCTCCCGGAC-3'
CD11a	5'-GCCGAGATCCC AGTACATGAA-3'	5'-ACTGTGATGTTGA CTCCTTCCTTCT-3'	5'-AGTGCTCCCCTTCA GCCAGCAACAA-3'
CD45	5'-TGATGATTTCTGG AGGATGATCTG-3'	5'-CACTTGTTCCTATT TCCTTCTTCACA-3'	5'-AAGCCACAGTCATTG TCATGGTCACTCG-3'

followed by counterstaining with hematoxylin. Stained samples were imaged using standard microscopy and assessed for alizarin red staining. Intracellular calcium concentration was measured using a commercially available kit (QuantiChrom™ Calcium Assay Kit, BioAssay Systems, Hayward, CA) in cell extracts collected on days 2, 3, 4, and 7 following induction. Protein content in the same cell extracts was determined using the Bradford protein assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Calcium concentration was expressed as μg Ca^{2+} /μg of total protein.

Chondrogenic induction: Pellet cultures were generated using 5 x 10^5 cells /pellet with processing as previously described [19]. Pellet cultures were maintained in medium consisting of high glucose DMEM containing 100μg/mL sodium pyruvate, 10 ng/mL TGF-β3, 100nM dexamethasone, 1x Insulin/Transferrin/Selenium (ITS+1) premix, 40 μg/mL proline and 25 μg/mL ascorbate-2-phosphate (all Sigma-Aldrich, Inc. St. Louis, MO). Medium was replaced twice weekly. Samples were collected on days 1, 3, 7, 14, 21 and 28 of culture. Pellets were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 4 micron sections. Matrix metachromasia was assessed with Safranin-O/fast green staining.

Statistical analysis: Gene expression data were categorized into four groups by culture duration: 1=less than one week; 2=one week; 3=two weeks; 4=three weeks or more. Mean fluorescence intensity data were categorized into 5 groups by culture duration: 1=2 days; 2=one week; 3=two weeks; 4= three weeks; 5=four weeks. Calcium/protein ratio data were categorized into one of four groups by induction duration: 1=control (no osteogenic induction); 2=48 hours of induction; 3=72 hours of induction; 4=one week of induction. Groups were compared using a One-Way ANOVA with a Tukey All-Pairwise Comparisons post hoc test. A p-value of <0.05 was considered significant.

Results

Cytology of bone marrow aspirate: Cellular counts and distribution were compared between whole bone marrow aspirate and following gradient density centrifugation. Density gradient cell isolation was highly effective in removing RBC from the bone marrow aspirate with only 0.07% of original RBC remaining after isolation (**Table 2.3**).

Approximately 70% of nucleated cells were also lost during processing. The monocytic lineage had the highest post-processing recovery at 47%, while the eosinophil lineage had the lowest recovery with only 5% of pre-processing numbers.

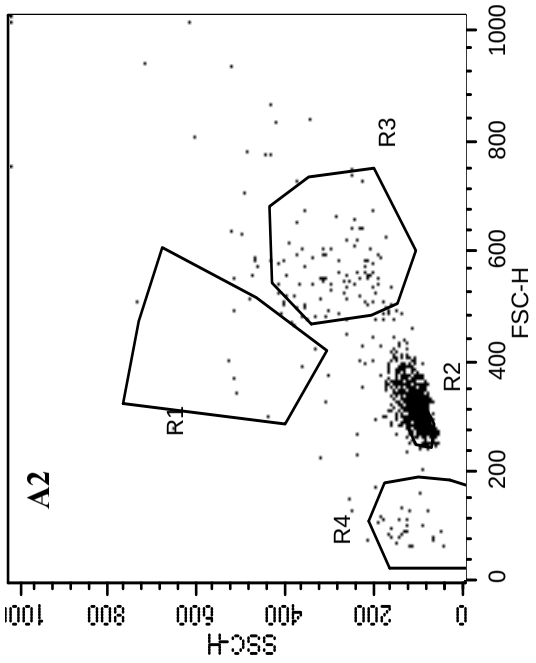
Validation of antibodies against equine peripheral blood and bone marrow cells. Equine CD44, human CD29, canine CD90, and equine CD11a/CD18 antibodies were confirmed reactive to equine molecules using flow cytometry analysis (**Figure 2.1** and **Figure 2.2**). The bovine CD45RB antibody had questionable reactivity with equine molecules. CD45RB data is included to demonstrate the importance of antibody validation, and the value of gene expression data in providing supporting evidence when protein expression detection is questionable.

To confirm reactivity of the antibodies to equine molecules, isolated peripheral blood neutrophils (**Figure 2.1, B**) lymphocytes (**Figure 2.1, C**) and monocytes (**Figure 2.1, D**) were tested. Results could then be used for comparison with fresh bone marrow cells of the same size and granularity (**Figure 2.2**). In addition, for cells of the monocytic lineage, bone marrow was cultured for two hours and the adherent cells were collected and compared to uncultured bone marrow cells of the same size and granularity (see **Figure 2.3**, 2 hour sample). There was increased mean fluorescence intensity for the CD44 and CD11a/CD18 molecules in both lymphocyte and monocyte lineage cells compared to uncultured bone marrow cells of the same size and granularity, confirming these antibodies were reactive to mononuclear cells.

Table 2.3 Bone marrow aspirate cell count and differential before and after density centrifugation cell isolation. Cell count is calculated to reflect a 60mL BMA sample. Mean (Min/Max) n=8.

Cell Type	**Pre-isolation (x 10^6) cells**	**Post-isolation (x 10^6) cells**	**% original post isolation**
Red blood cells	375,000 (299,000 / 427,000)	264 (45 / 565)	0.07
Red cell precursors	754 (77 / 1,660)	181 (24 / 438)	24.00
White cells and precursors	847 (463 / 1,340)	310 (106 / 747)	36.60
Neutrophils	590 (211 / 989)	216 (44 / 558)	36.61
Monocytes	42 (19/ 74)	20 (6 / 41)	47.28
Mitotic myeloid (lineage was not classified)	23 (2/ 63)	7 (2/ 15)	28.95
Lymphocytes (and precursors)	191 (106 / 266)	61 (27/ 112)	31.99
Eosinophils	15 (6/ 33)	1 (0 / 2)	5.16

Figure 2.1 Flow cytometric analyses of cell surface molecule expression in isolated peripheral blood cells. Dot plot distribution of uncultured peripheral blood cells isolated using A1) gradient density centrifugation or A2) carbonyl iron incubation followed by gradient density centrifugation. B-D) Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated areas (Regions 1, 2, and 3, respectively, for isolated peripheral blood cells). The R1 gate corresponds to the size and granularity of neutrophils; R2 lymphocytes; and R3 monocytes. The shaded curves represent negative isotype control staining. Open lines represent the labeling for the cell surface markers indicated. The expected labeling pattern for each marker is indicated in parenthesis. Note that CD44, CD29, and CD11a/CD18 are detected in all three regions, while CD90 is detected in R1 (neutrophils) and CD45RB is detected in only a small population of R2 (lymphocytes) and R3 (monocytes).


A2
R1
R2
R3
R4
SSC-H
FSC-H
0
200
400
600
800
1000

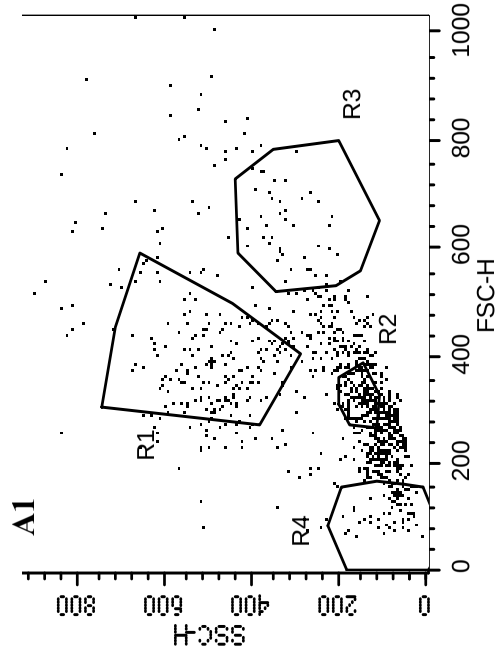
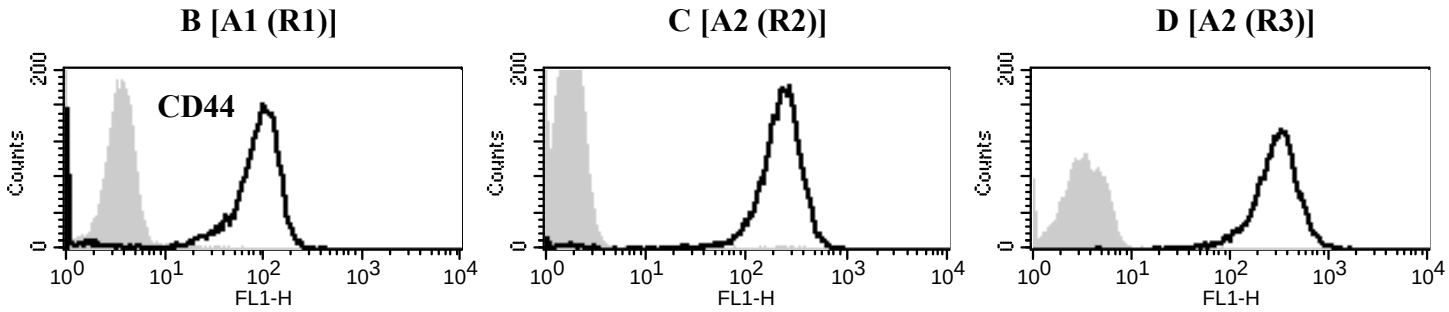
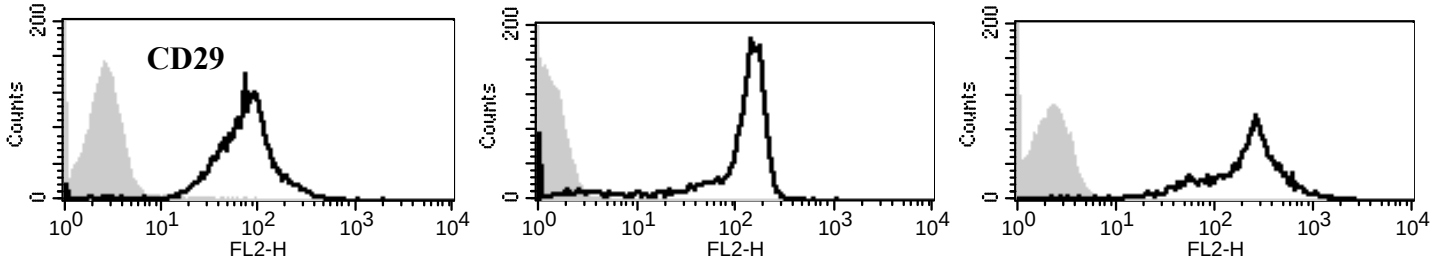

A1
R1
R2
R3
R4
SSC-H
FSC-H
0
200
400
600
800
1000

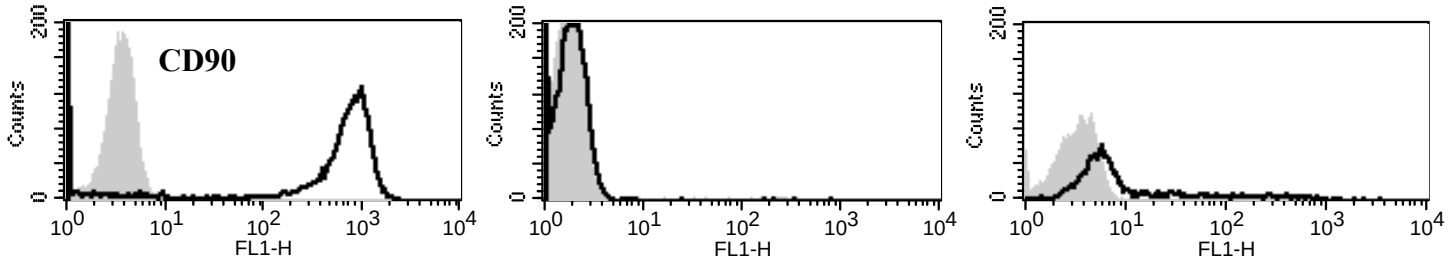
Figure 2.1 (Continued)



(Mature WBCs should be positive)

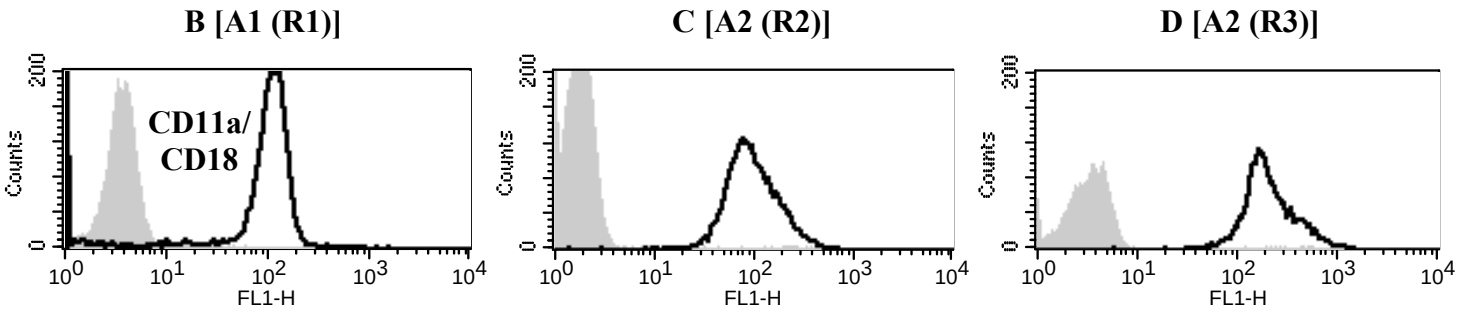


(All WBCs and platelets should be positive, with lower levels on neutrophils)



(Equine neutrophils should be positive)

Figure 2.1 (Continued)



(Mature WBCs should be positive)

(Neutrophil/lymphocyte populations should be positive)

Figure 2.2 Flow cytometric analyses of surface molecule expression in uncultured bone marrow cells. A) Dot plot distribution of uncultured bone marrow cells isolated using gradient density centrifugation B-E) Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated areas (regions 1, 2, 3 and 4, respectively). Cells in region 1 are equivalent in size and granularity to neutrophils; region 2, lymphocytes; region 3, monocytes; and region 4, red blood cells and platelets. The shaded curves represent negative isotype control staining; open lines represent the labeling for the cell surface markers indicated in the left-hand side. Note the variation in the CD44 and CD11a/CD18 expression in Region 2 gated cells between uncultured bone marrow (C) and peripheral blood isolated cells (see **Figure 2.1, C**). Also, the expression of these molecules in Region 3 is different between uncultured (D) and isolated peripheral blood cells (see **Figure 2.1, D**) or 2-hour cultured bone marrow cells (**Figure 2.3**). (WBC, white blood cell, RBC, red blood cell)

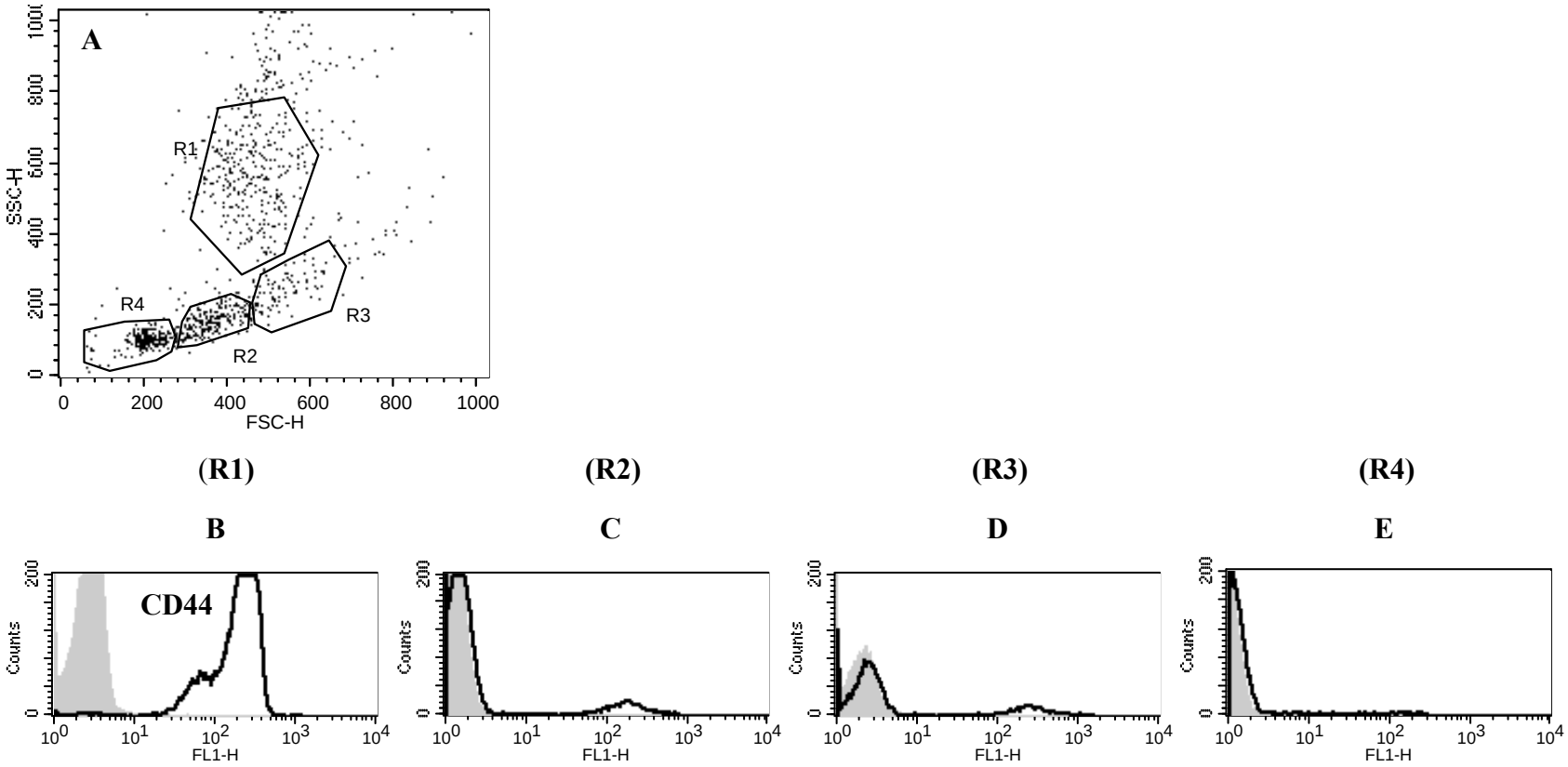

A
R1
R2
R3
R4
SSC-H
FSC-H
0
200
400
600
800
1000
(R1)
(R2)
(R3)
(R4)
B
C
D
E
CD44
Counts
FL1-H

Figure 2.2 (Continued)

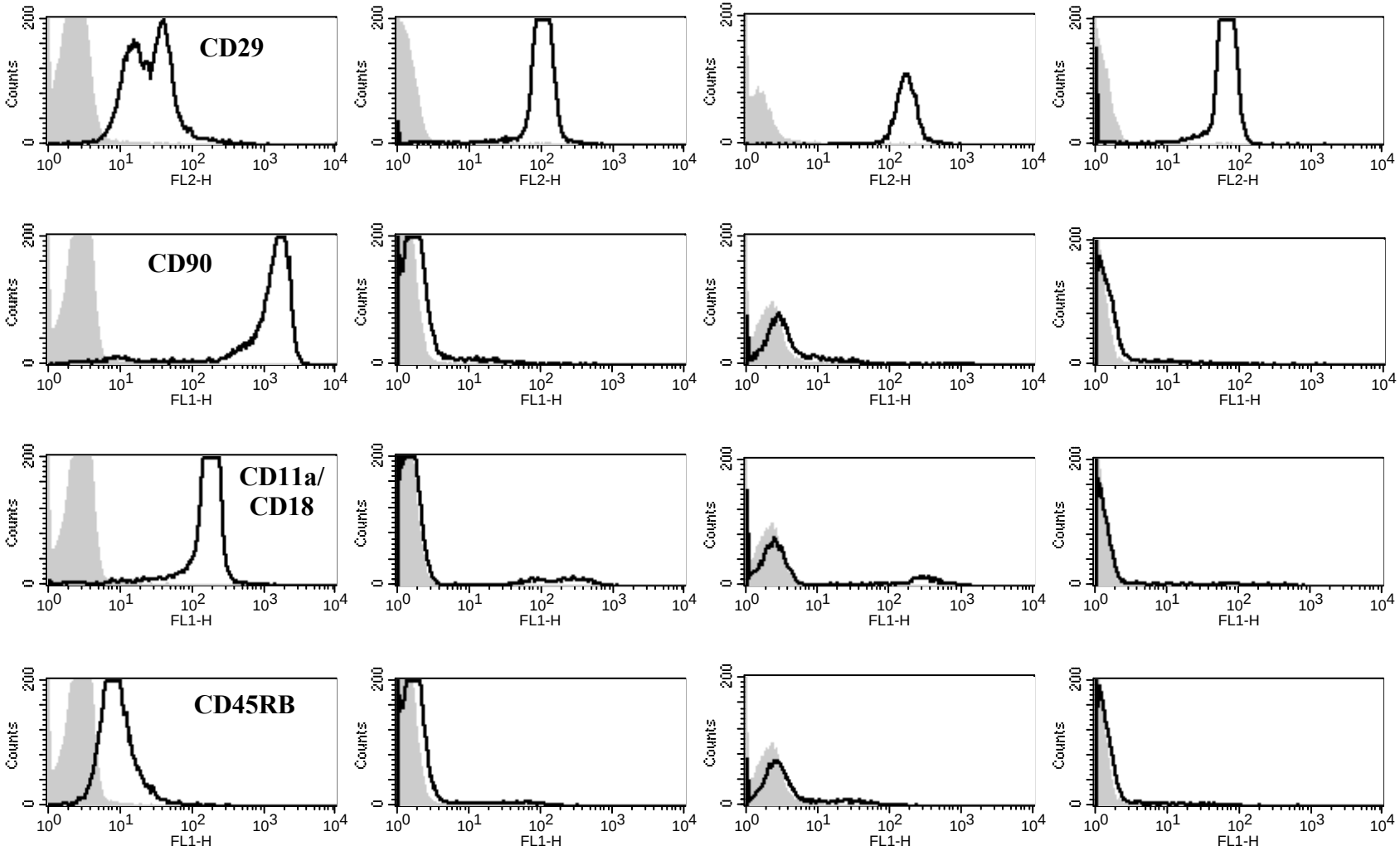


Figure 2.3 Flow cytometric analyses of cell surface molecule expression in bone marrow cells over increasing culture periods. **Part A**. Dot plot distribution of bone marrow cells isolated using gradient density centrifugation followed by culture of indicated duration. **Part B**. Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated area (Region 3). The shaded curves represent negative isotype control staining; open lines represent the labeling for the cell surface markers indicated in the left-hand side. At two hours, adherent cells gated in Region 3 displayed a molecular profile consistent with monocyte lineage, while later time points revealed changes, particularly in CD90 and CD11a/CD18 expression. At 48 hours, the presence of multiple cell types was evidenced by several peaks in antibody labeling for CD44, CD29 and CD90. By 14 days, when cells display a fibroblastic morphology in culture, putative MPCs were positive for CD44, CD29 and CD90, while negative for CD45 and CD11a/CD18. This staining pattern is retained throughout long term culture.

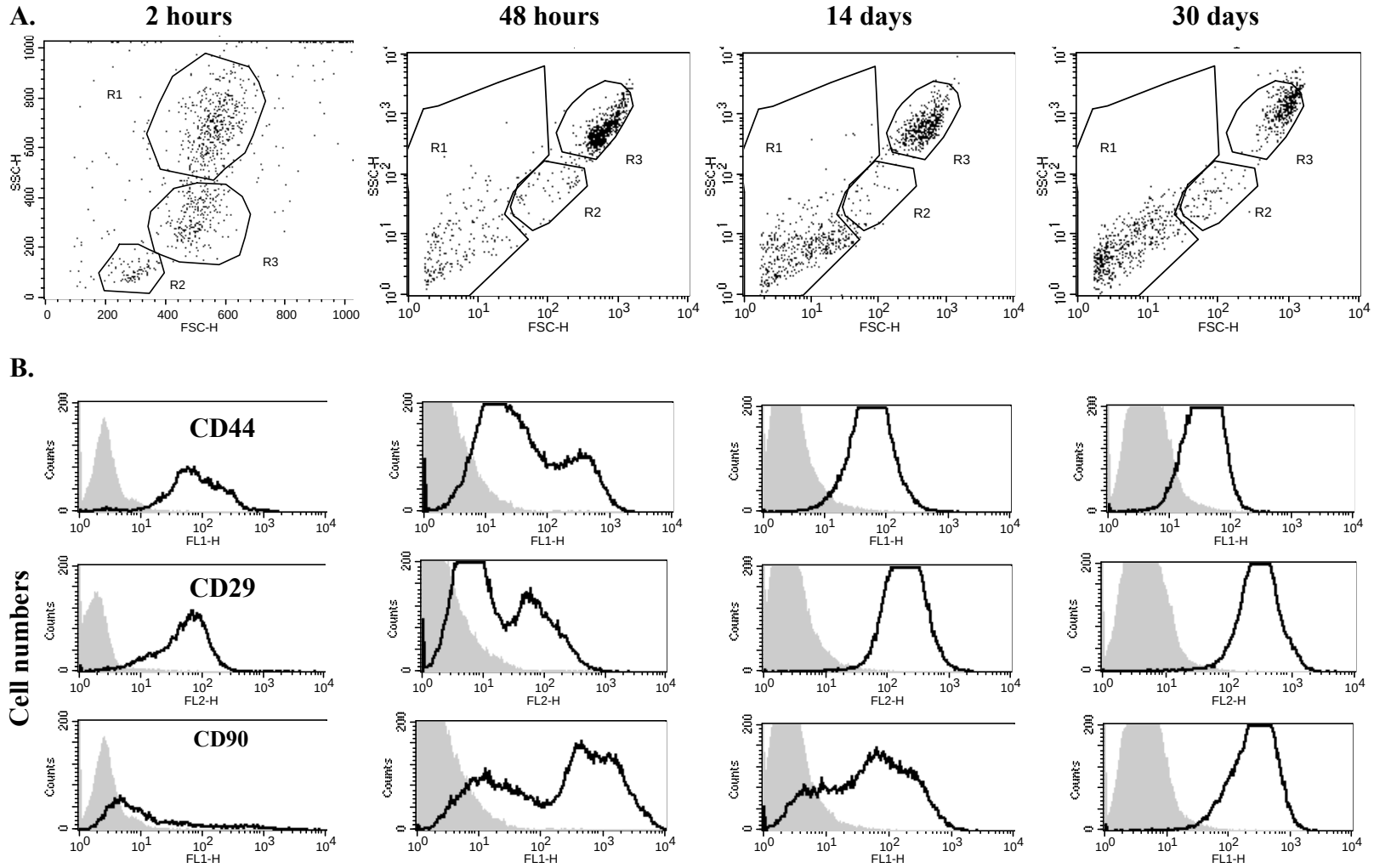
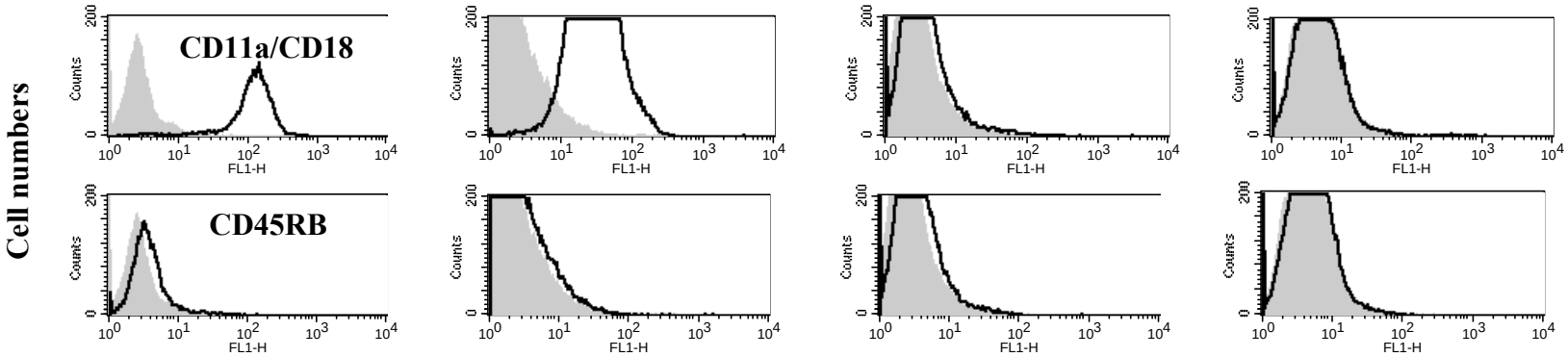

A.
2 hours
48 hours
14 days
30 days
R1
R2
R3
SSC-H
FSC-H
B.
CD44
CD29
CD90
Cell numbers
Counts
FL1-H
FL2-H

Figure 2.3 (Continued)

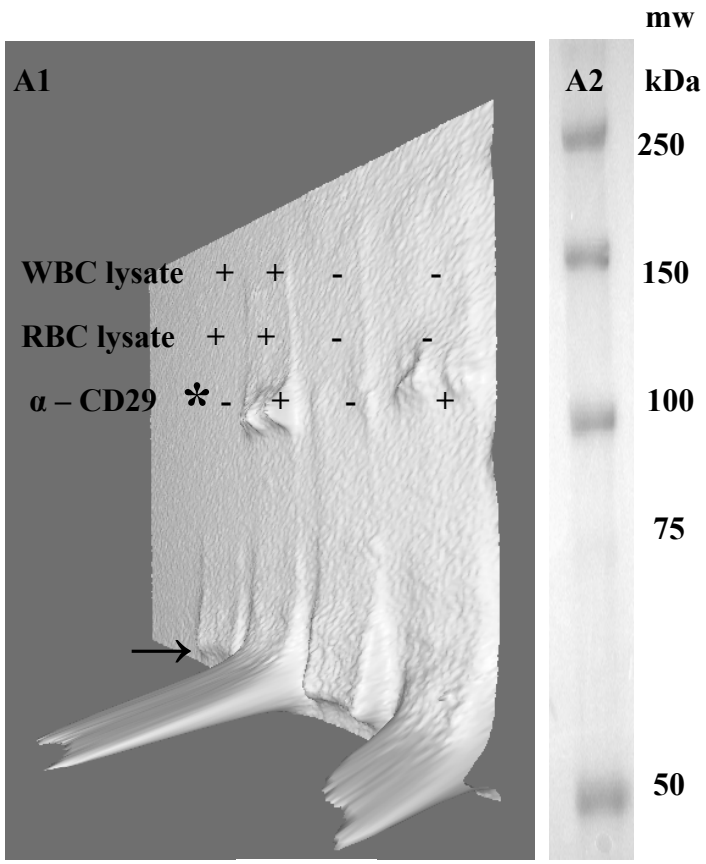


By analogy to peripheral blood cells, region 1 included cells of the neutrophil lineage, region 2 to the lymphocyte lineage, and region 3 to the monocytic lineage. Interestingly, CD44, and CD11a/CD18 antibodies were variably immunoreactive in freshly isolated bone marrow cells. Cells in region 1 were $CD44^{hi}$, $CD29^{hi}$, $CD90^{hi}$, $CD11a/CD18^{hi}$ and $CD45RB^{hi}$ (see **Figure 2.2, B**). Regions 2 and 3 contained bone marrow cells that were $CD44^{lo}$, $CD29^{hi}$, $CD90^{lo}$, $CD11a/CD18^{lo}$ and $CD45RB^{lo}$ (**Figure 2.2, C and D**), suggesting these regions contained cell types other than mature mononuclear cells. The monoclonal antibodies against equine MHC class I, equine MHC class II and equine CD3 labeled the expected cell populations, and were used as internal controls for subsequent flow cytometry assays

Other antibodies tested were found to be non-reactive to equine molecules or differed in expression from MPCs of other species. The specific lot of monoclonal antibody against equine CD13 and several human antibodies tested, including Stro-1, SSEA 1, SSEA 3, SSEA 4, and CD34 did not label equine cells using flow cytometry. No equine-specific positive controls, such as embryonic stem cells, were available to confirm the negative findings.

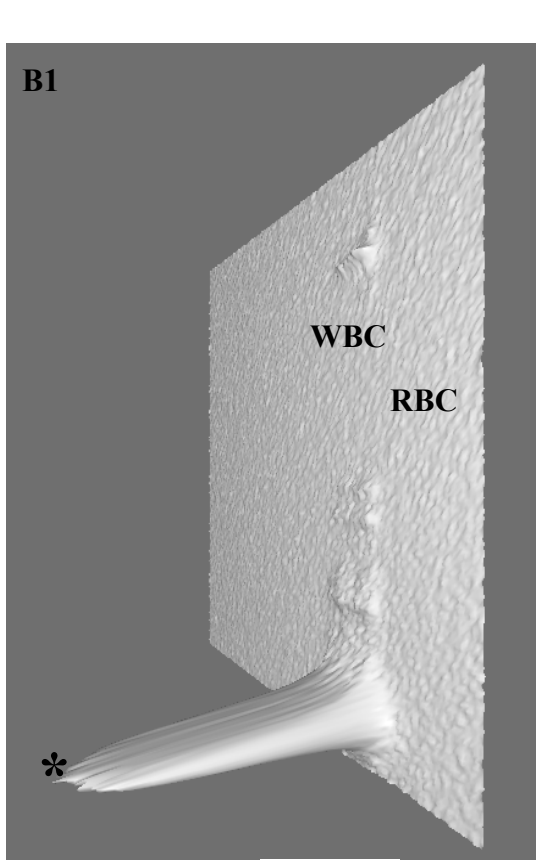
Validation of protein specificity: Western blot analysis alone was not clear to determine specificity of the CD29 antibody reaction with equine blood cells. No distinct band was detected on multiple attempts to analyze CD29 antibody binding. It was proposed that this antibody did not recognize the denatured protein. The Beckman Coulter CD29 antibody, clone 4B4LDC9LDH8, has been extensively used for immunoprecipitation in human cells [20]. Immunoprecipitation using clone 4B4LDC9LDH8 antibody followed by Western blot analysis with the Calbiochem clone 4B7-CP26 antibody confirmed the CD29 antibody (4B4LDC9LDH8) reacted with a single protein of approximately 130kDa (**Figure 2.4**). Western blot analysis was successful in demonstrating the CD90 antibody reacted with a protein of

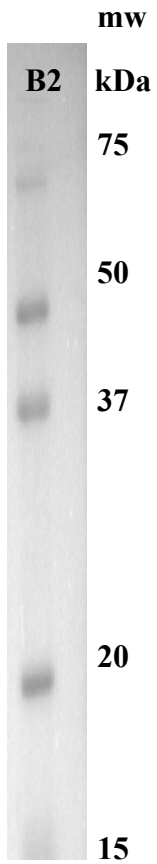
Figure 2.4 Western blot analyses to test the specificity of CD29 and CD90 antibodies in equine peripheral blood cells. **A1.** Three-dimensional image of a western blot following immunoprecipitation using anti (α)-human CD29 antibody (Beckman Coulter, clone 4B4LDC9LDH8) with equine blood cells and probing with α-human β1 integrin antibody (Calbiochem, clone 4B7-CP26). White blood cell (WBC) or red blood cell/platelet (RBC) lysates incubated with α-CD29 antibody retained a protein band, of approximately 130kDa (*). Arrow indicates the heavy chain of IgG. **A2.** Molecular size standards. **B1**. Three-dimensional image of a western blot of equine WBC or RBC lysate probed with an α-canine CD90 antibody (VMRD, clone DH24A). An approximately 17kDa protein band was detected in equine WBC lysate (*). **B2**. Molecular size standards.


A1
WBC lysate + + - -
RBC lysate + + - -
α – CD29 * - + - +

mw
A2
kDa
250
150
100
75
50

CD29


B1
WBC
RBC
*


mw
B2
kDa
75
50
37
20
15

CD90

appropriate size of 17kDa (**Figure 2.4**) in equine peripheral blood leukocytes, and was not detected in red blood cells or platelets. The CD45RB antibody did not react with a protein of expected size of ~150kDa on Western blot analysis. Instead, multiple poorly defined protein bands were noted of varying sizes (data not shown).

DNA content analysis to determine cell cycle stage of cultured bone marrow cells: DNA content analysis was useful for identification of the region of interest (Gate 3) on the dot plot of cultured MPC, and subsequent flow cytometry analysis was restricted to this cell population (**Figure 2.5**). Proliferating cultured bone marrow cells were identified in Gate 3 by the high number of cells in the S/G2/M phase based on their increased DNA content [17]. Cells in S/G2/M were consistently located only in this gate. In contrast, in Gate 1 (R1) and Gate 2 (R2), the majority of cells are presumed to be dying (subG0).

Cell surface marker expression in cultured bone marrow cells changes over time in culture: After two hours of culture, adherent mononuclear cells displayed an antibody labeling pattern of $CD44^{hi}$, $CD29^{hi}$, $CD90^{lo}$ and $CD11a/CD18^{hi}$, $CD45RB^{lo}$ (**Figure 2.3)**. By two days of culture, expression of CD11a/CD18 began to decrease, while CD90 expression was increased compared to expression levels at two hours. In Gate 3, there appeared to be a heterogeneous cell population as evidenced by multiple peaks in CD44, CD29, and CD90 mean fluorescence intensity. Over the next several days there was a population shift as cells with high CD90 expression decreased in population percentage compared to cells with lower mean fluorescence intensity for CD90 expression. On day seven, CD11a/CD18 cell surface expression further decreased, while CD90 expression began to increase following a trough in CD90 expression from days two through seven. By fourteen days, adherent cells in culture were $CD44^{hi}$, $CD29^{hi}$, and $CD90^{hi}$, $CD11a/CD18^{neg}$ and $CD45RB^{neg}$; these cells displayed a fibroblastic morphology

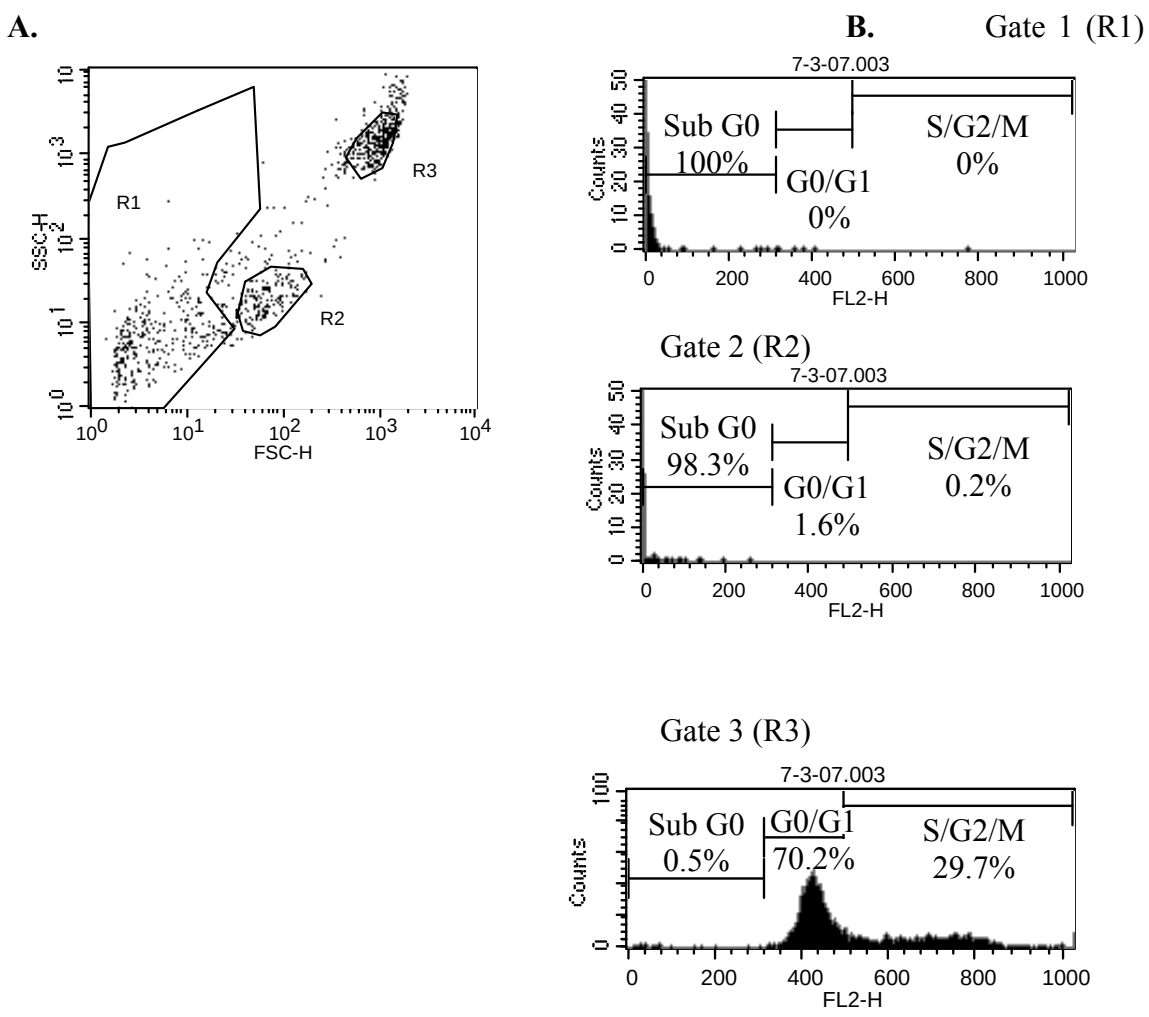


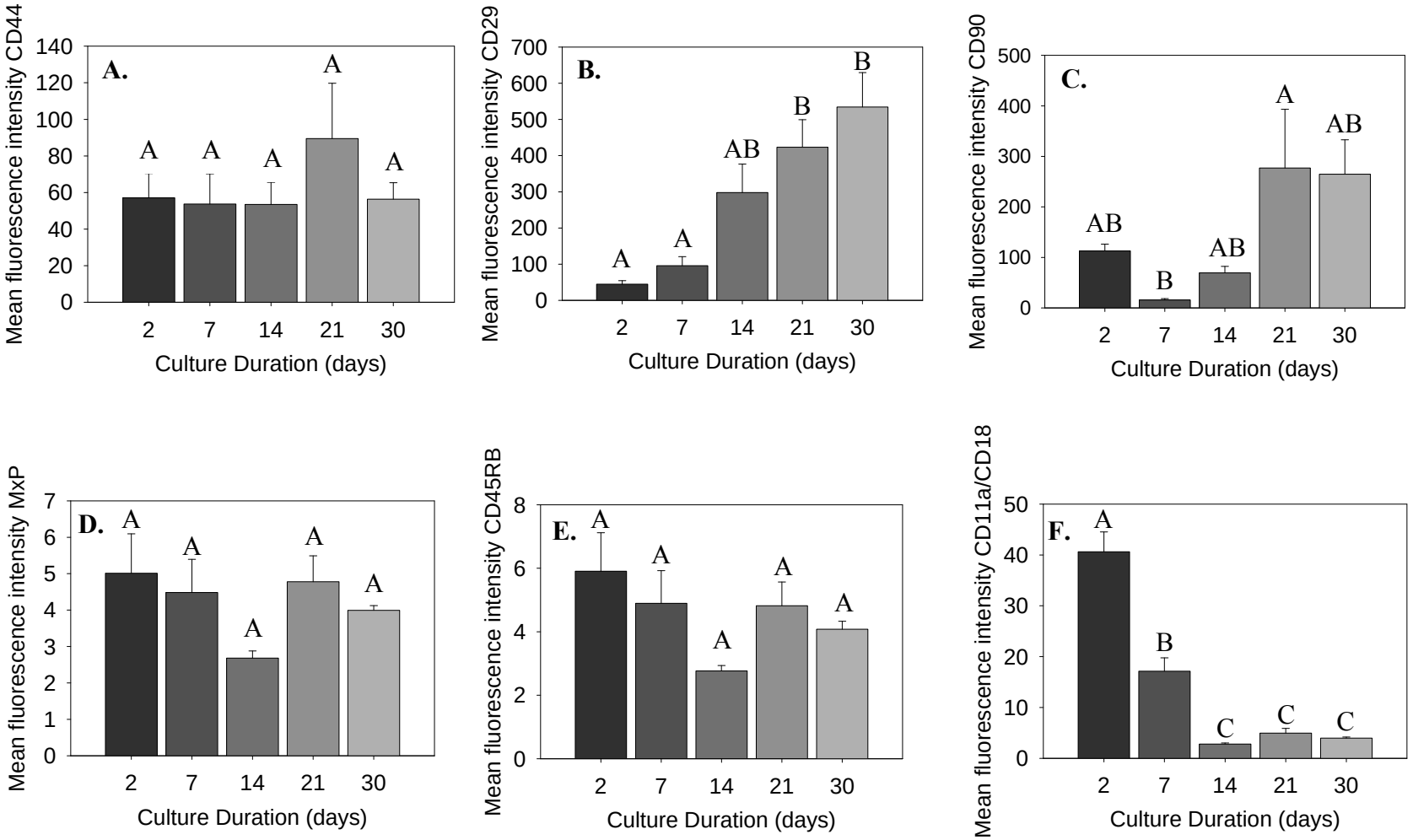
Figure 2.5 Cell cycle analysis of bone marrow cells in culture. (A) Flow cytometry dot plot (side scatter, SSC x forward scatter, FSC) demonstrates the distribution of three cell populations (R1, R2, R3) in bone marrow aspirate cultured for 14 days. (B) Flow cytometry histogram analyses of each gated area in Figure 2A reveals the cell cycle status according to their DNA content (stained with propidium iodide). In Gates 1 (R1) and Gate 2 (R2), the majority of cells are within sub-G0 and G0/G1, consistent with cell death. In Gate 3 (R3), cells are undergoing division, and 1/3 of cells are in S/G2/M phase. Therefore, the flow cytometric cell surface molecular analysis reported in Figure 3 was performed with cells gated in Region 3 (R3).

characteristic of MPC (data not shown). This pattern of molecule expression was retained at 21 and 30 days for all MPC samples. At all time points (2 hours, 2, 7, 14, 21, and 30 days), adherent bone marrow cells in culture showed a consistent labeling pattern with the results described above in all samples analyzed.

The mean fluorescence intensity varied by culture duration for several of the antibodies analyzed (**Figure 2.6**). The mouse anti-parvo virus negative control antibody ($P=0.19$) did not vary significantly over time. The mean fluorescence intensity of CD44 expression was also not significantly different over time ($P=0.57$). The mean fluorescence intensity of CD29 expression was significantly greater in cells cultured fourteen days or more compared to earlier time points ($P\leq0.001$). Mean fluorescent intensity for CD90 expression was significantly different ($P=0.01$) over time, with a decrease at seven days compared to other time points. The mean fluorescence intensity of CD45RB was not significantly different between time points ($P=0.11$), with all samples having minimal protein detection, comparable to the control negative antibody. Finally, CD11a/CD18 expression decreased significantly over time ($P\leq0.001$) in early samples compared to samples cultured fourteen days or more. Targeted analysis using flow cytometry revealed a number of significant differences in protein marker expression between bone marrow cells cultured for different time periods.

Gene expression in cultured bone marrow cells: Gene expression data followed a consistent pattern with cell surface protein expression at all culture time points. In samples where gene expression was high, the corresponding antibody mean fluorescence intensity was also detected at high levels. The same pattern was true for samples with low or undetectable levels of gene expression. There was little or no increase in mean fluorescence intensity for the corresponding molecule in these samples compared to the mean fluorescence intensity of the control negative antibody.

Figure 2.6 Flow cytometry analysis of mean fluorescence intensity over time in cultured bone marrow cells. The mean fluorescence intensity of selected markers was tested by one-way ANOVA and Tukey's All-Pairwise Comparisons (n=6 ± SE). There were significant ($P \leq 0.05$) differences between mean fluorescence intensity over time for several antibodies [CD29 (B), CD90 (C), and CD11a/CD18 (F)], but no significant differences for the CD44 (A), CD45RB (E), or the control negative MxP (D) antibodies. CD45RB data was included to demonstrate that mean fluorescence intensity using this antibody was similar to the control negative antibody.

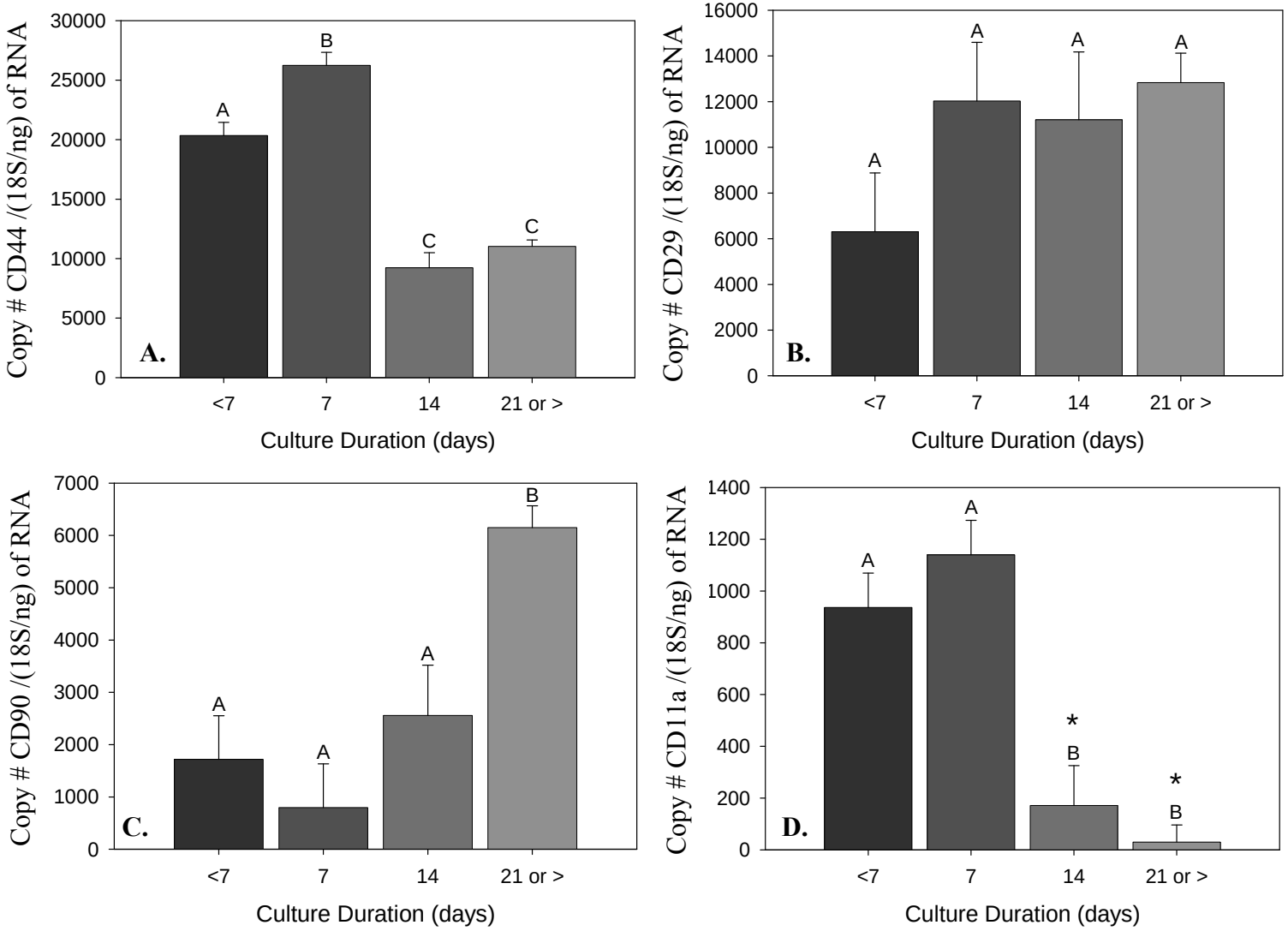

A.
Mean fluorescence intensity CD44
140
120
100
80
60
40
20
0
A A A A A
2 7 14 21 30
Culture Duration (days)
B.
Mean fluorescence intensity CD29
700
600
500
400
300
200
100
0
A A AB B B
2 7 14 21 30
Culture Duration (days)
C.
Mean fluorescence intensity CD90
500
400
300
200
100
0
AB B AB A AB
2 7 14 21 30
Culture Duration (days)
D.
Mean fluorescence intensity MxP
7
6
5
4
3
2
1
0
A A A A A
2 7 14 21 30
Culture Duration (days)
E.
Mean fluorescence intensity CD45RB
8
6
4
2
0
A A A A A
2 7 14 21 30
Culture Duration (days)
F.
Mean fluorescence intensity CD11a/CD18
50
40
30
20
10
0
A B C C C
2 7 14 21 30
Culture Duration (days)

Since the grouping of samples for statistical analysis varied between gene and protein expression, and the flow cytometry analysis could be targeted to the cell population of interest, slight differences were detected in gene expression data from the statistical results of mean fluorescence intensity reported above.

CD44 gene expression was present in all samples but changed over time (**Figure 2.7**). Early samples (≤ 7 days) had significantly more *CD44* expression compared to samples cultured for 14 days or more ($P < 0.001$). *CD29* gene expression was also high and present in all samples, but no significant differences were detected based on culture duration ($P=0.19$). Expression of *CD90* varied with culture duration and was significantly greater in cells cultured for 21 days or more compared to all earlier samples ($P < 0.001$). Gene expression of *CD11a* and *CD45* was present in all samples cultured 7 days or less. In contrast, samples cultured 14 days or longer failed to reach a C_T value with a threshold of 40 cycles in 60% of samples analyzed for *CD11a*, and 84% for *CD45*. For *CD11a*, early duration culture samples had significantly more expression than samples cultured 14 days or more ($P < 0.001$). CD45 gene expression levels also significantly decreased with increasing culture duration ($P<0.001$).

Differentiation assays: Tri-lineage differentiation capacity of cultured bone marrow cells was confirmed through *in vitro* adipogenic, osteogenic and chondrogenic assays. Following induction, histochemical staining for adipogenesis and osteogenesis in short term (≤7days) assays and longer duration chondrogenic induction (up to 4 weeks) were consistent with previous reports of MPC differentiation potential [2]. In the description by Pittenger et al., lipid vacuoles were detected within 48 hours of induction under adipogenic conditions, calcium accumulation continued for at least three weeks, and Safranin O staining increased over the four week time period under chondrogenic conditions.

Figure 2.7 Gene expression kinetics in cultured bone marrow cells. The gene expression of selected markers over increasing culture durations was tested by one-way ANOVA and Tukey's All-Pairwise Comparisons (n=6 ± SE). There were significant ($P \leq 0.05$) differences between gene expression (copy number) over time for most genes [CD44 (A), CD90 (C), CD11a (D) and CD45 (E)], but no significant difference for CD29 (B) gene. For CD11a and CD45, the majority of samples cultured 14 days or longer did not reach a C_T value. Samples with gene expression below the detectable limit (*) were assigned a quantity of 1 to permit statistical analysis. Schematic relative gene expression changes during the establishment of MPC cultures are shown in F.


Copy # CD44 /(18S/ng) of RNA
30000
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Culture Duration (days)
Copy # CD29 /(18S/ng) of RNA
16000
14000
12000
10000
8000
6000
4000
2000
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A
B.
<7
7
14
21 or >
Culture Duration (days)
Copy # CD90 /(18S/ng) of RNA
7000
6000
5000
4000
3000
2000
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0
A
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B
C.
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14
21 or >
Culture Duration (days)
Copy # CD11a /(18S/ng) of RNA
1400
1200
1000
800
600
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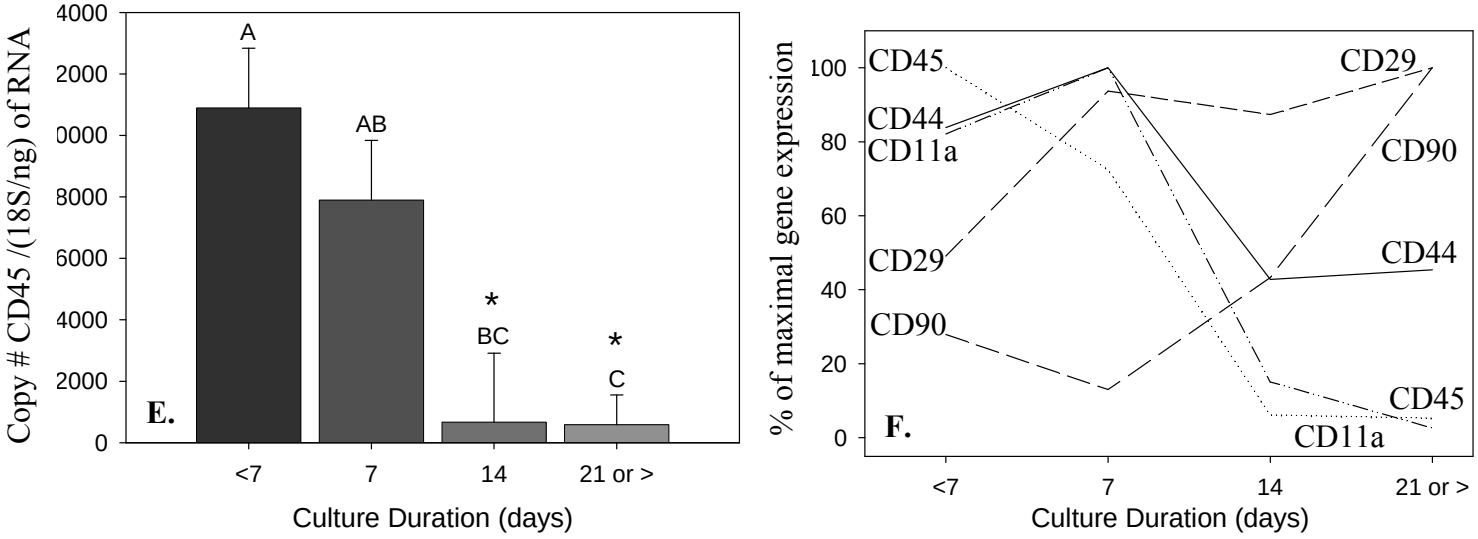
14
21 or >
Culture Duration (days)

Figure 2.7 (Continued)

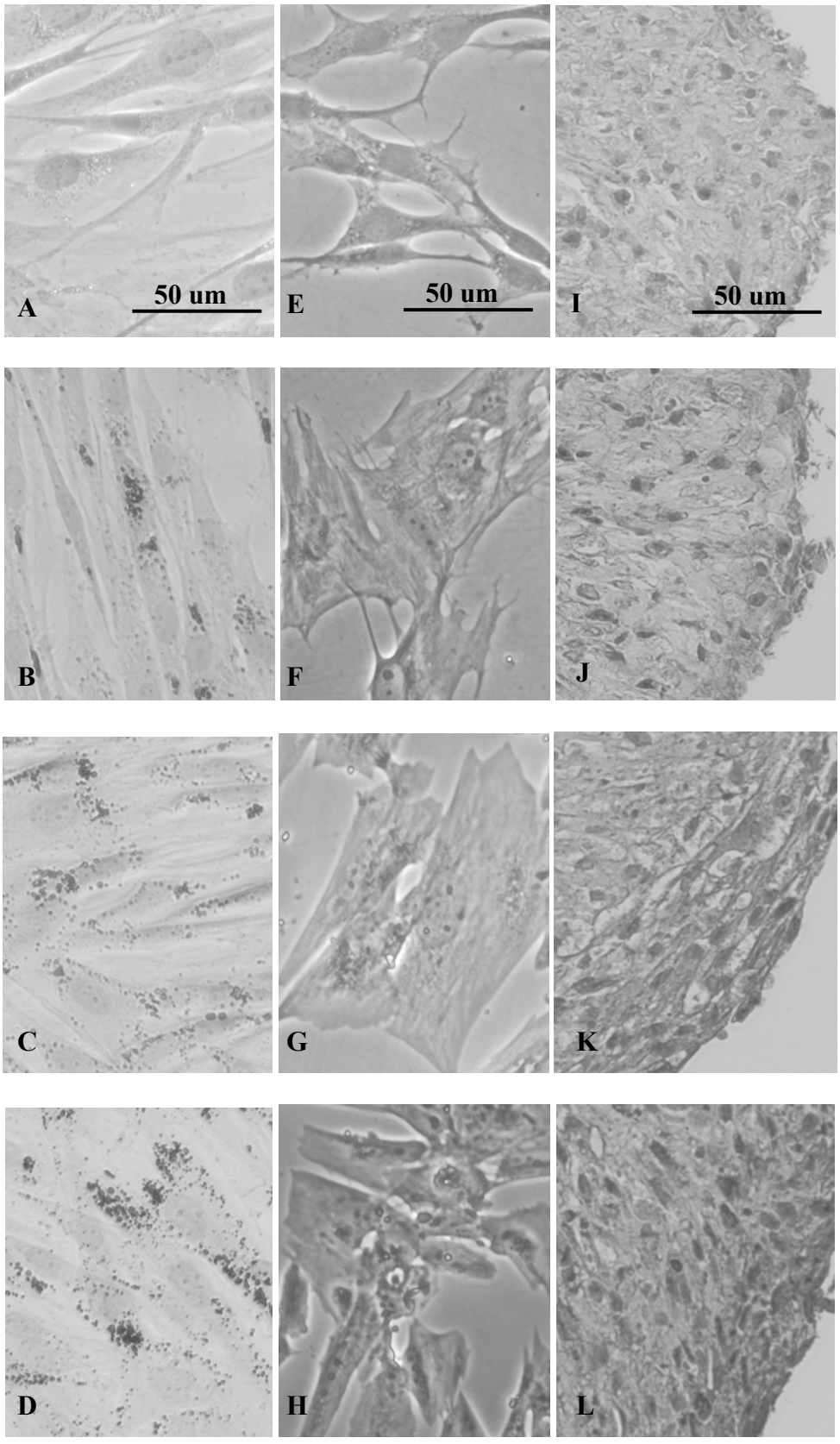


Under adipogenic culture conditions, MPCs accumulated a large amount of lipid vacuoles in their cytoplasm, while control samples had no appreciable staining for lipid (**Figure 2.8**). Osteogenic culture conditions induced a change in cell morphology from fibroblastic to a stellate or cuboidal shape within 7 days (**Figure 2.8**). Early calcium accumulation was evidenced by alizarin staining in the first 7 days of culture. Calcium accumulation in MPCs following osteogenic induction was significantly increased by day 7 compared to earlier time points and control samples as assessed using calcium/protein ratios of the samples (P=0.003). Control cells had 0.347± 0.038 μg Ca^{2+} /μg total protein which increased to 0.490 ± 0.120 μg Ca^{2+} /μg total protein within 24 hours of osteogenic induction. By 72 hours, content was 0.756 ± 0.226 μg Ca^{2+} /μg total protein and by 7 days the ratio was 1.502 ± 0.467 μg Ca^{2+} /μg total protein, a 3-6 fold increase in relative calcium compared to control conditions. During chondrogenic induction, an increase in pellet size was noted with increasing culture duration over the 28 day culture period. The pellets also had progressively more matrix metachromasia as evidenced by enhanced staining with Safranin-O stain with increased culture duration (**Figure 2.8**).

Discussion

In this study temporal changes in cell surface protein and gene expression in MPC in culture were demonstrated. Based on the literature, it was anticipated that established equine MPC cultures would be negative for CD45RB and CD11a/CD18, and positive for CD44, CD29, and CD90 (Thy-1)[2][21]. Protein expression data in early cultured (2 hour) bone marrow mononuclear cells comprised $CD44^{hi}$, $CD29^{hi}$, and $CD11a/CD18^{hi}$ positive cells, with a smaller population of $CD90^{lo}$ and $CD45RB^{lo}$ positive cells. Established cultures of MPC were robustly positive for CD44, CD29 and CD90, becoming negative for CD11a/CD18 and CD45RB. Gene expression data

Figure 2.8 Differentiation assays using cultured equine MPC. A-D lipid induction with Oil-Lipid-O staining, E-H osteogenic induction with Alizarin staining, I-L chondrogenic induction with Safranin O/fast green staining. A,E control MPC; B,F 24 hour induction; C,G 72 hour induction; D, H 7 day induction. I 3 day induction; J 1 week induction; K 2 week induction; L 4 week induction.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followed the same pattern, in which established cultures retained expression of *CD44*, *CD29* and *CD90*, whereas levels of *CD11a* and *CD45* dropped below the level of detection by 14 days of culture. The molecules detected by these antibodies were found to have protein and gene expression patterns consistent with results of protein expression in cultured MPCs of other species [2, 4, 22]. Equine MPCs had positive expression of CD44, CD29, and CD90 and negative expression of CD11a/CD18 and CD45RB similar to flow cytometry analysis of expanded human bone marrow cells by Pittenger et al. Sung et al. noted CD44 and CD29 were present in expanded mouse MPCs and CD45 was absent. Although other studies have found similar antibody labeling in established cultures of 14 days or more [21], none have specifically documented changes in gene and protein expression over time from isolation through two weeks and beyond.

In bone marrow samples cultured for 48 hours, a mixed population of cells was still present despite selecting for strongly adherent cells by vigorously washing the plates to prior to sample collection. This was evidenced by multiple peaks in CD44, CD29 and CD90 mean fluorescence intensity and side scatter distribution (data not shown) of cells in flow cytometry. At least two populations of cells expressing CD90 in different mean fluorescence intensities were detected at the protein level. This molecule is highly expressed in equine neutrophils. Neutrophils are known to enter apoptosis spontaneously within 24 to 36 hours of culture [2], so they would be progressively removed during subsequent media changes, and the relative levels of *CD90* gene and protein expression of remaining cells should decrease proportionally during early culture. Over time, the cells expressing CD44, CD29 and CD90 became more homogeneous (see **Figure 2.3**, 48 hours versus 14 days) based on the flow cytometric histogram analyses. Expression of CD44 or CD29 molecule alone is not useful to determine putative MPCs because they are not unique to MPCs. Although these

molecules are not included in the minimal criteria for defining human multipotent mesenchymal stromal cells, they may play a role in equine characterization studies as part of a combination of positive and negative markers, since expression of the molecules is sustained in long term culture [23].

Gene expression data was especially useful to confirm negative results when analyzing CD45RB cell surface protein expression, as CD45RB expression was weakly/inconsistently detected in isolated peripheral blood leukocytes and bone marrow cells when tested by flow cytometry. The bovine CD45RB antibody used in this study had previously been validated for the horse [21]. Nevertheless, protein detection using flow cytometry can be affected by variability in the reagents (monoclonal antibody product lot or secondary conjugated-antibodies) or instrument settings used. Therefore, validation of reagents and standardized data collection and analysis are important for consistency in results. I have included the CD45RB data specifically to demonstrate the importance of reagent validation. Gene expression data can be a useful, complementary tool for cellular immunophenotyping when protein expression data is inconsistent, or reagents are not readily available, as it was for CD45RB. In the future, with adequate reagents to the equine species, the protein expression for CD45 can be confirmed.

An interesting observation of this study was the difference in cell surface molecule expression between fresh bone marrow mononuclear cells and peripheral blood leukocytes, or short term (2 hour) cultured bone marrow mononuclear cells for CD44 and CD11a/CD18. To my knowledge, other studies that have analyzed and/or antibody sorted fresh marrow did not account for this difference in protein expression [24, 25]. Mitchell et al. [26] reported initially low levels of CD44 protein expression in uncultured cells, which increased during successive passages. The lack of antigen identification on freshly isolated cells from the bone marrow can be misleading since

cells may not be sufficiently mature to express proteins characteristic of their lineage especially when trying to classify hematopoietic versus non-hematopoietic cells. Therefore, I would suggest a short duration culture (e.g. two hours) to better classify adherent bone marrow cells based on their molecular expression of hematopoietic markers (e.g. CD11a/CD18).

Lack of expression of Stro-1, SSEA1, SSEA3, SSEA4, and CD34 in isolated bone marrow and cultured cells could not be verified due to unavailability of equine specific reagents, and the lack of reactivity of human reagents to horse molecules. Lack of positive controls prevents the validation of these reagents for equine molecules. The lack of cross-reactivity of several human antibodies to equine molecules is not surprising given the recent work by Ibrahim S. et al. [27] which reported only 14 out of 379 monoclonal human antibodies tested cross-reacted in a cell-type specific manner with equine leukocytes. The findings of these studies emphasize the importance of rigorous testing with controls when using xenogenic antibodies.

Density gradient centrifugation of equine bone marrow aspirate was successful in removing nearly all of the red blood cells and allowed for analysis of the mixed mononuclear cell fraction in fresh samples using flow cytometry. Approximately 30% of nucleated cells were retained for analysis which is identical to that of human bone marrow aspirate using the same technique [28]. The number of mononuclear cells in equine aspirates following isolation were also proportionate to human bone marrow mononuclear cell counts on a per ml of aspirate basis, and 3-5X more total mononuclear cells were harvested/isolated from equine since 3-5X the volume of aspirates were collected. Equine bone marrow samples provided sufficient cell numbers to allow analysis at multiple early time points.

An accepted characteristic of MPC is the highly proliferative nature of this cell type [2]. Previous studies have not attempted to target the dividing cell population when analyzing MPC markers. Cell cycle analysis of cultured bone marrow cells was a simple way to identify the dividing population. Characterization of surface protein expression for this specific population may be more accurate than ungated analysis, as cells from the non-dividing populations are removed from analysis. In actively dividing cultures, I have noted approximately 40-65% of the total cells are located in the region of interest (Region 3). As cultures senesce, I have noted a drop in the percentage of cells located in Region 3 to 10-30% (data not shown) with a shift in cellular distribution towards Regions 1 and 2 of the dot plot; and decreases in mean fluorescence intensity of CD90 and CD44 expression in Region 3. Not only does analysis of cellular markers in Region 3 focus on the dividing cell population, relative cell distribution between the three regions also reflects the overall proliferative activity of the culture. In this study, targeted analysis using flow cytometry to assess mean fluorescence intensity of cells only in the region of interest allowed for additional significant differences to be identified between culture time periods compared to analysis of gene expression alone.

Much work remains to be done in the full characterization of the equine MPC. This study presents a preliminary molecular profile using both gene and protein expression levels of bone marrow nucleated cells from isolation to one month in culture. Understanding the early changes in cultured bone marrow cells may promote identification of cellular markers unique to early MPC's and help distinguish them from hematopoietic and other cell types. My results suggest that freshly isolated cells from bone marrow aspirate do not express surface proteins uniformly due to varying stages of cellular maturity. This may lead to less accurate cell sorting when using freshly isolated bone marrow cells. For example, use of the cell surface markers CD44 or CD11a/CD18 to sort freshly isolated bone marrow cells may incorrectly

select immature hematopoietic cells expressing low levels of these proteins. Short term culture allows selection of adherent, more mature cells, leading to more accurate classification of cell lineage and the potential for mesenchymal differentiation. Taking advantage of changes in marker expression during culture establishment may be beneficial for enhanced isolation of MPC from bone marrow aspirate or other tissue sources.

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CHAPTER 3

ANALYSIS OF CD14 EXPRESSION LEVELS IN PUTATIVE MESENCHYMAL PROGENITOR CELLS FROM EQUINE BONE MARROW

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Abstract

A long-term goal of my dissertation research is to identify both positive and negative MPC cell surface markers to facilitate isolation of equine MPCs in future cell sorting studies. One of the reported features of mesenchymal progenitor cells (MPCs) in humans and other species is lack of expression of the cluster of differentiation (CD) marker CD14, also known as the lipopolysaccharide receptor (LPS-R). There are no reports of either upregulation or downregulation of CD14 gene or protein expression in equine MPCs. My aim was to evaluate CD14 expression patterns in equine bone marrow as a potential negative MPC marker. My hypothesis was that cells negatively selected by CD14 expression (non-hematopoietic cells) would enrich MPC colony formation compared to the CD14 positive hematopoietic and unsorted fractions. Bone marrow aspirate was obtained from twelve horses and processed by density gradient centrifugation. Fresh and cultured cells were analyzed by a combination of flow cytometry and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). In fresh marrow aspirate, cells of early hematopoietic origin did not uniformly express CD14 protein or other hematopoietic specific markers such as CD11a/CD18, complicating the distinction of hematopoietic versus non-hematopoietic cells when assessed using flow cytometry. It was proposed that many freshly isolated hematopoietic cells from the bone marrow were not yet expressing cell surface proteins normally associated with mature peripheral blood cells. Short term (2 day) culture allowed enrichment of adherent bone marrow cells. The enriched cell types included adherent myeloid hematopoietic cells, which now expressed cell surface proteins associated with peripheral blood cells, and putative MPCs. The distinction between hematopoietic and non-hematopoietic cell lineages, using a differentially expressed protein, could easily be made following 2 days of culture. A cell sorting experiment was performed to exploit the difference in CD14 protein expression

between adherent hematopoietic (CD14 positive) and mesenchymal (CD14 negative) lineages to enrich for MPC colony formation. Magnetic activated cell sorting (MACS) was used to separate bone marrow cells from six horses based on CD14 expression after 2 days of culture. Flow cytometry and RT-qPCR were used to evaluate sort efficiency and analyze cultured cells over time. Quantitation of MPC colony formation in the sorted cell fractions were assessed at day 7 and compared to each other and the unsorted cell fraction. Cells positively selected for CD14 expression were significantly more likely to form MPC colonies than both unsorted and negatively selected cells ($P \leq 0.005$). Further, MPCs from all three (CD14 positive, negative and unsorted) fractions maintained low levels of CD14 expression in long term culture and could upregulate *CD14* gene and protein expression when stimulated with lipopolysaccharide (LPS). An additional experiment demonstrated that the equine CD14 cell surface molecule was trypsin labile on MPCs, offering a plausible explanation for the discrepancy with negative CD14 protein results reported in other species. Only certain lineages of hematopoietic cells (e.g. monocytes, macrophages, dendritic cells, activated B lymphocytes and to a lesser extent neutrophils) are known to express CD14. By definition, MPCs of other species are thought to be non-hematopoietic in lineage because they lack expression of specific hematopoietic molecules such as CD14. The expression of CD14 by equine MPCs raises the question whether these cells are truly derived from a non-hematopoietic lineage. It is possible they are a differentiated descendant of a CD14 positive cell, or they may have simply been misclassified previously as negative for CD14 expression.

Introduction

Mesenchymal progenitor cells (MPC) are found in bone marrow and other tissues, and are defined using a number of criteria [1]. MPC are adherent to tissue culture plastic and can differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro*. Besides these features, in humans, these cells are defined by cell surface molecule expression of the cluster of differentiation (CD) molecules CD105, CD73, and CD90 and the lack of expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR in culture expanded cells [1]. Classification of MPCs using the CD cell surface phenotype has been utilized as supporting evidence of a unique cell population that can be distinguished from hematopoietic and other cell lineages.

The CD nomenclature system classifies monoclonal antibodies by the epitope they recognize on the cell surface [2, 3]. Human Leukocyte Differentiation Antigen (HLDA) workshops have summarized the antibody clusters grouped by the CD nomenclature system, and many more surface molecules continue to be defined, with only an estimated 10-20% of all surface molecules defined to date [4]. If two or more specific monoclonal antibodies bind to the molecule of interest, a CD designation is assigned. If only one monoclonal has been shown to bind, a provisional indicator "w" is included in the name. More than 350 designations have been classified for humans. Although the CD classification system was initially developed for leukocytes, a number of other cell types, including stromal cells, have been phenotyped based on the presence or absence of CD molecules [5].

Characterization studies of established human MPC cultures using differentiation assays, gene expression analysis, and cell surface protein markers have been performed for nearly a decade [6]. Most studies evaluate MPC cell surface markers and gene expression after population expansion in culture in order to obtain sufficient cell numbers for analysis [7-10]. However, there are reports of conflicting

results in MPC marker protein expression patterns when comparing phenotypes of freshly sorted MPCs to expanded MPCs [11, 12]. These studies suggest that the phenotype of MPCs is dynamic during isolation and culture processes.

One of the defining features of MPCs in humans and other species is lack of expression of the CD14 marker, also known as the lipopolysaccharide receptor (LPS-R) on their cell surface. The CD14 protein epitope is an important component of the innate immune system for detection of lipopolysaccharide (LPS). The LPS-R associates in a complex (53-55kDa) with an adaptor protein known as MD-2 and the toll-like receptor 4 (TLR4) signaling proteins. The CD14 receptor can only bind LPS when lipopolysaccharide-binding protein (LBP) is also present. The LPS-R has a molecular weight of 40kDa when separated from the complex. Only certain lineages of hematopoietic cells (e.g. monocytes, macrophages, dendritic cells, activated B lymphocytes, and to a lesser extent neutrophils), are known to express CD14. Therefore, CD14 would be a candidate cell surface marker to differentiate between adherent (primarily myeloid) CD14 positive hematopoietic cells and MPCs, which should be negative for CD14 expression.

Despite many reports that suggest the CD14 cell surface molecule is absent in MPCs, a few reports have suggested otherwise. In 2003, Kuwana et al. described selection of CD14 positive cells from human peripheral blood which could then be differentiated into numerous mesenchymal tissues including fat, bone, skeletal muscle and cartilage *in vitro* [13], with a later study demonstrating differentiation into cardiomyocytes [14]. This group referred to the CD14 positive population of interest as monocyte derived mesenchymal progenitors (MOMP). Other groups have named peripheral blood cells with similar phenotype and differentiation capacity programmable cells of monocytic origin (PCOM) [15]. Pufe et al. demonstrated that these cells were able to form collagen type II producing chondrocytes *in vitro*. Using

an antibody against Mac-1 (an epitope specific to monocyte/granulocyte lineage cells; also known as CD11b/CD18), Sera et al. demonstrated that adipocytes could be derived from hematopoietic cells of monocyte/macrophage lineage [16]. Incidentally, these studies all used ethylenediaminetetraacetic acid (EDTA) for cell harvest prior to analysis using flow cytometry, which will become important in later discussion with respect to trypsinization. In another study, putative MPCs from cord blood have been isolated with the initial cell phenotype of CD45+,CD105+, CD14+, CD49a+, CD49f+, CD44+ and CD34-. Later, the culture passaged cells were CD45-, CD14-, CD34- and weakly CD105+ [17].

Most MPC characterization studies have utilized trypsin, a serine protease, for cell harvest prior to flow cytometric analysis. Trypsin cleaves peptide chains at the carboxyl side of lysine or arginine. It is commonly used to disrupt adherent cells to permit cell passage or harvest in tissue culture applications. Trypsin has previously been shown to cleave a number of cell-membrane molecules, including CD14 [18]. It is possible that trypsin has interfered with detection of the CD14 epitope on candidate cells in previous MPC studies.

In this study, my aim was to evaluate CD14 expression patterns in equine bone marrow as a potential negative MPC marker. My hypothesis was that bone marrow cells negatively selected for CD14 expression would enrich for MPC colony formation. Fresh and cultured cells were analyzed by a combination of flow cytometry and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). I proposed that a mouse anti-equine CD14 antibody could be used to separate the adherent CD14 positive hematopoietic cell fraction (primarily neutrophil and monocyte lineages) from the putative MPCs using magnetic activated cell sorting (MACS). The MACS technique was used to separate bone marrow cells from six horses based on CD14 expression after two days of culture into one of three fractions

1) unsorted, 2) CD14 positive, and 3) CD14 negatively selected. Flow cytometry and RT-qPCR were used to evaluate sort purity and analyze cultured cell fractions over time. Quantitation of MPC colony formation was assessed at day seven by comparing the numbers of colonies in each fraction relative to initial cell numbers plated following MACS. In addition, I tested the responsiveness of putative MPCs to LPS stimulation and evaluated the equine CD14 epitope to determine if it was trypsin labile as assessed using flow cytometry and RT-qPCR.

Materials and Methods

Study design: The mouse anti-equine CD14 antibody (clone 105; B. Wagner, Cornell University) was tested for reactivity and specificity with the equine CD14 cell surface molecule. Whole blood (30 mL) was collected from two horses for antibody validation of reactivity. Blood samples were drawn into preservative free heparin to a final concentration of 33 units/mL. The blood was processed prior to flow cytometry analysis using density gradient centrifugation to remove the majority of red blood cells as previously described [19]. Isolated peripheral blood cells were analyzed to evaluate reactivity of the antibody with neutrophil, lymphocyte, and monocyte populations as assessed by flow cytometry. For CD14 specificity analysis, whole cell lysates were prepared from fresh peripheral blood leukocytes and from red blood cells with platelets from two additional horses. Immunoprecipitations followed by Western blot analysis were performed to determine if the CD14 antibody bound a protein of the expected size (40kDa) based on previous literature, protein size similarity to other species, or predicted equine sequences. A 15% SDS-PAGE gel was used to resolve the immunoprecipitated products. Following protein transfer, the PVDF membrane was probed with an antibody known to recognize human CD14 (mouse anti-human CD14, clone biG 10, Biometec, Griefswald, Germany) in Western blot analysis.

Subsequently, cell surface expression of CD14 and a panel of previously validated antibodies were analyzed in uncultured bone marrow cells using flow cytometry (n=12). Following 2 hours of culture, both adherent and non-adherent fractions of bone marrow cells were harvested and compared to freshly isolated bone marrow cells using flow cytometry (n=5). Bone marrow cells were cultured and adherent cells were harvested on 2, 7, 14, 21, and 30 days for analysis of cell surface protein and gene expression (n=7). In a later experiment, bone marrow cells were isolated, cultured and harvested for magnetic activated cell sorting (MACS) at 2 days of culture (n=6). Cells from the three groups (unsorted, CD14 positive, or CD14 negatively selected) were returned to culture at a plating density of 20,000 cells/cm^2. Colony formation was assessed at day seven of culture and compared between groups. A colony was defined as a cluster of fifty or more cells. Responsiveness of MPCs to LPS stimulation (0, 1, 5, 10 ng/mL of media) in a sample cultured 21 days and sensitivity to trypsin (n=4) were tested in established MPC cultures (21 days or more) and assessed by flow cytometry and RT-qPCR. All procedures were performed in compliance with institutional guidelines for research on animals.

Bone marrow aspirate collection and cell isolation: Cells from bone marrow aspirate were used to assess changes in CD14 expression over time. Bone marrow aspirate was withdrawn from the sternabrae of twelve horses (6 males and 6 females, age range 6 months - 20 years) under standing sedation with xylazine hydrochloride (0.55 mg/kg IV) and local anesthesia using 2% lidocaine hydrochloride (10 mL/site). Samples were collected in preservative free heparin (American Pharmaceutical Partners Inc, Schaumburg, IL) to a final concentration of 33 units/mL.

Aspirate (60 mL) from each horse was diluted to 180 mL total volume using phosphate buffered saline + 0.5% bovine serum albumin (PBS +BSA). The white blood cell fraction of the sample was enriched and the majority of red blood cells were

removed by layering each 30 mL aliquot of dilute sample on Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) for density gradient centrifugation, as described for antibody validation. Samples were resuspended in 50 mL MPC culture media (see below) prior to cell counting using a hemocytometer. Approximately 2-9 x 10^8 bone marrow mononuclear cells (BMMNC) were obtained per sample using this method. A portion (~10 x 10^6 cells) of the uncultured bone marrow aspirate samples from all twelve horses were analyzed using flow cytometry.

Samples from some horses (n=5) were used only for antibody validation and were not cultured. The remaining samples (n=7) were subsequently cultured as described below. Later, bone marrow aspirates from an additional six horses were processed as described above, cultured for 2 days, and utilized for MACS cell separation with the CD14 antibody and subsequent quantification of colony formation, flow cytometry, and RT-qPCR analysis.

MPC expansion in culture: BMMNCs were plated onto 10 cm diameter tissue culture plates at a density of approximately 300,000 cells/cm^2 (20 x 10^6 cells/plate). Cells were cultured at 37°C in a 5% CO_2, 95% air atmosphere at 5% humidity. Cells were cultured in media containing Dulbecco's Modified Eagle's medium (DMEM, glucose at 1000 mg/L), 2mM L-glutamine, penicillin (100 units/mL), streptomycin (100 units/mL), basic fibroblastic growth factor (bFGF, 1 ng/mL) and 10% fetal bovine serum. One-half of the media (5 mL) was removed at 24 hours of culture and replaced with fresh media. Subsequently, media were exchanged every 72-96 hours. At sub-confluence of 70-90%, cells were passaged 1:3 using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, San Diego, CA) and plated at a density of 6,000-10,000 cells/cm^2. Approximately 10 x 10^6 cells from each sample was analyzed by flow cytometry for cell surface protein expression at two hours and

on days 2, 5, 7, 14, 21 and 30 of culture. Cells were analyzed at these time points to evaluate the changes in cell surface proteins over time.

Flow cytometry analysis: Cells were pelleted in aliquots containing 1 x 10^6 cells and labeled for CD14 and other cell surface molecules known to vary in expression on bone marrow cells over time in culture using monoclonal antibodies from a panel previously validated for the horse [20]. Cells were treated with a twenty minute blocking step using 10% normal goat serum in FACS-Buffer (phosphate buffered saline containing 2.5% fetal bovine serum). The cells were pelleted, washed with FACS-Buffer and pelleted again. Cell pellets were resuspended in unconjugated primary monoclonal antibody and incubated for 45 minutes at 4°C. Cells were then washed, a second fluorescent-conjugated goat anti-mouse IgG or IgM antibody [Fluorescein isothiocyanate (FITC) conjugated AffiniPure Goat Anti-Mouse IgG (H+L) or IgM μ Chain Specific, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA] was applied to the unconjugated primary antibodies, and the samples were incubated for an additional 45 minutes at 4°C. All primary monoclonal antibodies were labeled with FITC-conjugated secondary antibody (read at FL1). Cells were resuspended in FACS-Buffer and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with a 488μm argon laser and BD Cell Quest™ analysis software (BD Biosciences, San Jose, CA). Cells not treated with antibody, and cells exposed to mouse anti-parvovirus antibody and FITC secondary antibodies were used as negative controls. The settings for the flow cytometric analyses determined less than 2% positive cells for the control antibodies. Data was collected on 1x10^5 cells for each sample regardless of size and granularity to prevent bias in gating.

For culture expanded cells, flow cytometric analysis was performed on days 2, 7, 14, 21, and 30 following isolation. Supernatant was removed and adherent cells

were lifted from the plate using Accumax® solution (1 mL/15cm^2) to prevent damage to cell surface proteins and avoid cellular clumping. Cells were processed and analyzed by flow cytometry as described above, except dot plot settings were adjusted to a logarithmic scale in the cultured cells to include large, granular cells. Flow cytometric analysis of cell surface molecule expression was performed in the gate determined to contain dividing cells based on the results from the propidium iodide DNA staining assay as previously described [20].

RNA extraction and One-Step Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR): Gene expression analysis was included to confirm negative protein results and account for kinetic changes in transcription and translation. At the same time points (with the exception of the fresh and two hour samples) when cells were analyzed by flow cytometry, RNA was extracted from approximately 1-3 x 10^6 cells of the corresponding samples using either Trizol® (Life Technologies, Invitrogen, Carlsbad, CA) or the 5 Prime Perfect Pure RNA® extraction kit (5 Prime Inc, Gaithersburg, MD) according to the manufacturers' directions. RT-qPCR was performed to provide supporting evidence that *CD14* gene expression levels were consistent with cell surface CD14 protein expression at the different time points and later following stimulation of MPCs with LPS. *CD14* gene expression was also compared between MPC sorted cells harvested with trypsin or Accumax® solutions to confirm CD14 gene expression in these samples was consistent with other MPC samples cultured for a similar time period. RNA quantity and quality were determined using a Nanodrop® spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE), and visualization of 18 and 28S bands on 0.8% agarose gels. A portion of the *CD14* gene was cloned and agreed with previously reported data (AF200416).

Total RNA was reverse transcribed and amplified using the One-Step RT-PCR technique and the ABI PRISM 7900 HT Sequence Detection System **(**Applied Biosystems, Foster City, CA). The primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software Version 2.0b8a (Applied Biosystems, Foster City, CA). The *CD14* probe and primers were designed using equine specific sequences published in Genbank, and sequenced in our laboratory. The *18S* ribosomal subunit was used to normalize gene expression. The 2^{-ddCt} method was used for statistical analysis. A sample from unsorted bone marrow cells cultured 14 days was used as the calibration sample. The *18S* gene had the following primers and probe: Forward 5'-CGGCTTTGGTGACTCTAGATAACC-3'; Probe FAM (5')-CCATGGT AGGCACAGCGACTA-TAMRA (3'); and Reverse 5'-TCGAACGTCTGCCCTATC AACTTTCGAT-3'. *CD14* had the following primers and probe: Forward 5'-TACGTGCGCTCGGGTACTC-3'; Probe FAM (5')-CGCCTCAAGGAACTGACGC TGGA-TAMRA (3'); Reverse 5'-CATCGTGCCGGTTACCTCTAG-3'.

Magnetic activated cell sorting: Bone marrow aspirate was collected from six horses and processed as described above. Following mononuclear cell isolation, bone marrow cells were cultured 48 hours using routine expansion conditions. At 48 hours, the plates were washed twice with 5 mL of media to remove nearly all non-adherent cells prior to harvest using Accumax® cell dissociation solution (1 mL/15cm^2). Adherent cells were consolidated and resuspended in 500 μL of chilled and degassed MACS buffer which was composed of 0.5% bovine serum albumin in phosphate buffered saline with 2 mmol EDTA to prevent clumping. To remove any cell clumps prior to antibody labeling, samples were gravity filtered over MACS® pre-separation filters (Miltenyi Biotec, Inc, Auburn, CA). Following a cell count, approximately one-third of the sample was removed as the unsorted fraction for RNA extraction, flow

cytometry and culture. The remaining two-thirds of the sample was pelleted and resuspended in MACS buffer and mouse anti-equine CD14 antibody (90 μL buffer + 10 μL of antibody/10^7 cells), as this is the dilution used for flow cytometry. The primary antibody was incubated with cells for 10 minutes at 4°C. The labeled samples were washed twice with 2 mL of MACS buffer/10^7 cells followed by 5 minute centrifugations at 500 x g. Next, cell pellets were resuspended in 80 μL MACS buffer + 20 μL of rat anti-mouse IgG MACS microbeads /10^7 cells. Samples were incubated for 15 minutes at 4°C and then were washed with 2 mL of MACS buffer/10^7 cells followed by 5 minute centrifugation at 500 x g. The column, fit with a new pre-separation filter, was placed in the magnetic field and primed using 3 mL of chilled, degassed MACS buffer. A 20 gauge needle was affixed to the end of the column to slow flow of liquid through the column and prevent washout of positive cells. The pellet was resuspended in 500 μL of MACS buffer and placed over the primed pre-separation filter and column. The CD14 negative fraction was collected as the flow-through portion of the sample. The column was allowed to drain until the flow-through stopped dripping between all steps. Three washings with 3 mL of MACS buffer each were used to rinse additional negative cells from the column. The CD14 positive fraction of the sample was collected following removal of the column from the magnetic field. The column was loaded with 5 mL of MACS buffer and a plunger was used to force positive cells from the column into a collection tube. Positive and negative fractions were counted and the samples divided for RNA extraction, flow cytometry analysis, and culture as described above for the unsorted fraction.

Flow cytometry to assess sort purity: For the unsorted fraction, approximately 6 x 10^6 cells were collected for flow cytometry analysis using a previously validated panel of monoclonal antibodies and the CD14 antibody as previously described (see **Flow cytometry analysis**; page 87). The positive and negative sorted fractions had 2 x 10^6

cells allocated for flow cytometry analysis. 1 x 10^6 cells of each sorted fraction remained unlabeled with fluorescent secondary, the other 1 x 10^6 cells were labeled with goat anti-mouse IgG FITC secondary antibody and analyzed as previously described.

Colony formation assay: For all three fractions (CD14 positive, CD14 negative, and unsorted) 1.15 x 10^6 cells were collected for quantitative analysis of colony formation following one week of culture. A plating density of 20,000/cm^2 in 6 well plates was used. Routine culture commenced as described above. On day 7 of total culture (since bone marrow aspiration) the numbers of colonies present in each fraction were counted. A colony was defined as a cluster of 50 or more fibroblastic cells. Colony numbers were compared between the unsorted and CD14 positively and negatively selected fractions.

CD14 gene expression analysis between CD14 sorted and unsorted cells over time: *CD14* gene expression data was compared over time between unsorted, positive and negative selected cells using the 2^{-ddCt} method with an unsorted bone marrow sample cultured 14 days used for the calibrator sample as described above. The first time point when sufficient MPC numbers were available in three fractions was 14 days of culture for at least half of the horses (n=3), later time points had sufficient MPC numbers for all horses (n=6).

Analysis of CD14 expression in cultured MPCs in response to LPS stimulation: Bone marrow cells from one horse were isolated, cultured and MACS sorted as described above. At 21 days, a portion of the CD14 positive fraction and the unsorted fraction were incubated overnight with LPS (026:B6, L2762; Sigma-Aldrich, Saint Louis, MO) at varying dosages (0, 1, 5, or 10 ng/mL media). Samples of treated and control cells were collected for flow cytometry analysis of CD14 protein expression

and RNA extraction for RT-qPCR analysis of *CD14* gene expression. Assays on samples were performed as described above.

Analysis of cell surface marker expression in response to trypsinization: Bone marrow cells from four horses were isolated, cultured and MACS sorted as described above. At 30 days of culture, approximately 10 x 10^6 cells from each fraction (CD14 positive, CD14 negative or unsorted) were harvested using Accumax® cell dissociation solution as previously described. An additional 10 x 10^6 cells from each fraction were harvested following five minutes of incubation at 37°C using 0.25% trypsin in Hanks Balanced Salt Solution (HBSS) with divalent cations. 2 x 10^6 cells from each sample were used for RNA extraction and subsequent RT-qPCR (to verify *CD14* expression was present at comparable expression levels between fractions). The remaining 8 x 10^6 cells were used for flow cytometry analysis with the same panel of antibody markers as previously described.

Statistical analysis: Gene expression data for the initial unsorted cells were categorized into four groups by culture duration: 1=less than one week; 2=one week; 3=two weeks; 4=three weeks or more. Colony formation data were categorized into three groups by cell sorted fraction: 1= unsorted; 2=CD14 positive; 3=CD14 negative (data was blocked by horse). The gene expression data for analysis of sorted versus unsorted cells were categorized by culture duration: 1=two weeks; 2= three weeks; 3= four or more weeks. LPS cell stimulation data were categorized by cell fraction 1= unsorted; 2= CD14 positive, and LPS dose: 1= 0 ng/mL; 2= 1, 5, or 10 ng/mL LPS. A One-Way ANOVA, blocked by horse and Tukey's All-Pairwise Comparisons post hoc was used to compare the percentage of positive cells as assessed by flow cytometry between cell dissociation solutions (1=Accumax; 2=trypsin). The 2^{-ddCt} method was used for statistical analysis of *CD14* gene expression. The *18S* gene was used to normalize gene expression. Groups were compared using a One-Way ANOVA with a

Tukey All-Pairwise Comparisons post hoc test. A p-value of <0.05 was considered significant.

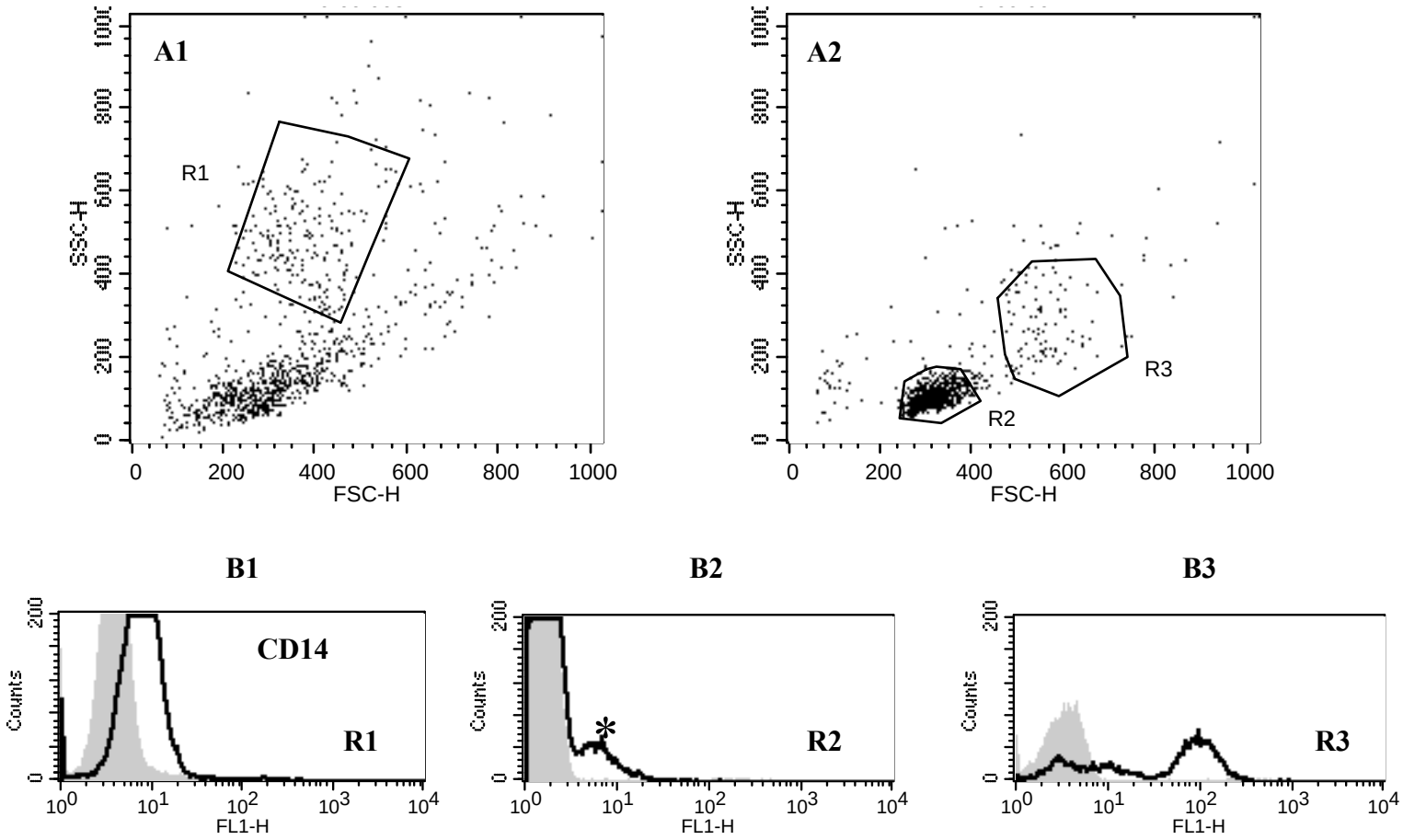
Results

Validation of antibody binding to the CD14 molecule on equine peripheral blood cells: Equine CD14 antibody was confirmed reactive to equine molecules on blood cells as assessed by flow cytometry analysis (**Figure 3.1**). There was low mean fluorescence intensity detected for CD14 in the neutrophil population (**Figure 3.1, B1**) and high mean fluorescence intensity in the monocyte population (**Figure 3.1, B3**) as expected. A small population of cells in the lymphocyte gate (**Figure 3.1, B2)** had low mean fluorescence intensity which likely represent the activate B lymphocyte population; however double labeling would be required to confirm that these cells are B lymphocytes.

Validation of CD14 specificity in equine peripheral blood cells: Western blot analysis alone did not clearly determine specificity of the CD14 antibody reaction with equine blood cells. No distinct band was detected on multiple attempts to analyze CD14 antibody binding. It was proposed that this antibody might not recognize the denatured protein. The BiG10 antibody (catalog #021-1c.2, Biometec, Greifswald, Germany) has been extensively used for immunoprecipitation in human cells. Immunoprecipitation using the mouse anti-equine CD14 antibody (clone 105) followed by Western blot analysis with the BiG10 antibody confirmed the CD14 antibody (clone 105) reacted with a single protein of approximately 40kDa (**Figure 3.2**).

Flow cytometric analysis of cell surface marker expression in bone marrow cells freshly isolated and cultured for two hours: There were no discernable differences in mean fluorescence intensity between freshly isolated cells and cells which were

Figure 3.1 Flow cytometric analyses of cell surface molecule expression of CD14 in freshly isolated peripheral blood cells. Dot plot distribution of uncultured peripheral blood cells isolated using A1) gradient density centrifugation or A2) carbonyl iron incubation followed by gradient density centrifugation. B1-B3) Histogram analysis of mean fluorescence intensity of CD14 cell surface molecule expression in the gated areas (Regions 1, 2, and 3, respectively, for isolated peripheral blood cells). The R1 gate corresponds to the size and granularity of neutrophils from A1; R2 lymphocytes from A2; R3 monocytes from A2; the shaded curves represent negative isotype control staining. Open lines represent the mean fluorescence intensity for CD14. Note that neutrophils (B1) have low mean fluorescence intensity for CD14 expression while monocytes (B3) have high mean fluorescence intensity. The small population of lymphocytes (B2*) which have low mean fluorescence intensity, likely represent the activated B lymphocyte population; however, double labeling would be required to confirm that these are B lymphocytes.



(Neutrophils should be low positive, lymphocytes should be negative with the exception of activated B cells and monocytes should be positive)

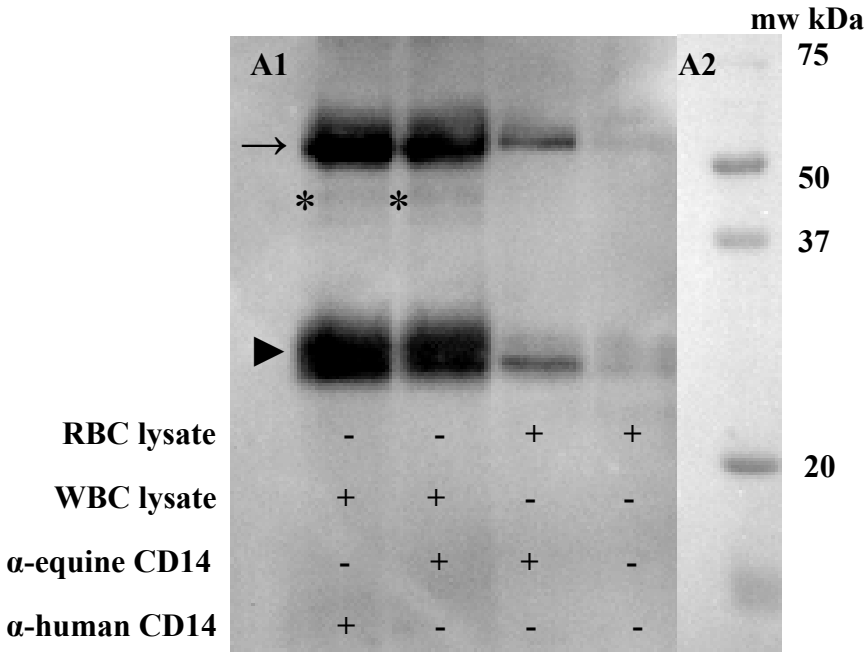


Figure 3.2 Western blot analyses to test the specificity of CD14 antibody in equine peripheral blood cells. **A1** Western blot following immunoprecipitation using either anti(α)-equine CD14 antibody (B.Wagner, Cornell University, clone 105) or mouse anti-human CD14 antibody (BiG10, catalog #021-1c.2, Biometec, Greifswald, Germany) with equine blood cells and probing with BiG10 antibody. White blood cell (WBC) or red blood cell/platelet (RBC) lysates incubated with both α-CD14 antibodies retained protein bands, of approximately 40kDa (*). Arrow (→) indicates the heavy chain of IgG; arrowhead (►) indicates the light chain of IgG. **A2** Molecular size standards.

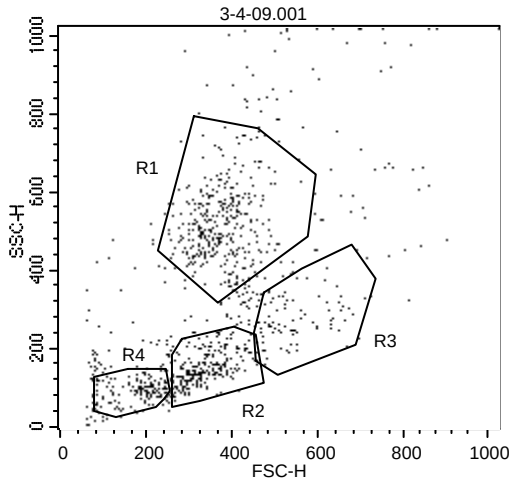
non-adherent after two hours of culture (**Figure 3.3** Parts A and B). There was a slight shift in population distributions between some regions (e.g. there were slightly higher percentages of cells in Region 2 in non-adherent cells compared to freshly isolated cells). In contrast, bone marrow cells, which were adherent following two hours of culture, had a unique dot pot distribution (Part C).

There were no notable differences detected in molecule expression in Region 1 between all markers analyzed for the three groups. There were dramatic differences in cell surface expression for all CD markers analyzed on cells in Regions 2 and 3 between freshly isolated or 2 hour non-adherent cells compared with adherent cells. cultured for 2 hours (Part D). Only a small population of cells was positive for CD44 expression in Regions 2 and 3 initially (and after two hours in the non-adherent cell fraction). In contrast, after two hours of culture, the few remaining Region 2 cells and a large number of Region 3 adherent cells were nearly all positive for CD44. Cells were brightly positive for CD90 expression for Region 1 in all three fractions, consistent with the expression profile of neutrophils. Cells in Region 2 were negative for CD90 expression in all fractions, consistent with the expression profile of lymphocytes. Most adherent cells in Region 3 were low positive in CD90 expression following culture and with a small population with higher levels of expression (*); this result is consistent with a mixture of two cell populations (the lower level would be more typical for the pattern of expression by monocytes and the higher mean fluorescent intensity is consistent with the pattern for neutrophils). Multiple populations when gates include overlapping cell populations in the interfaces. CD11a/CD18 expression was once again bright in all three fractions for cells in Region 1.

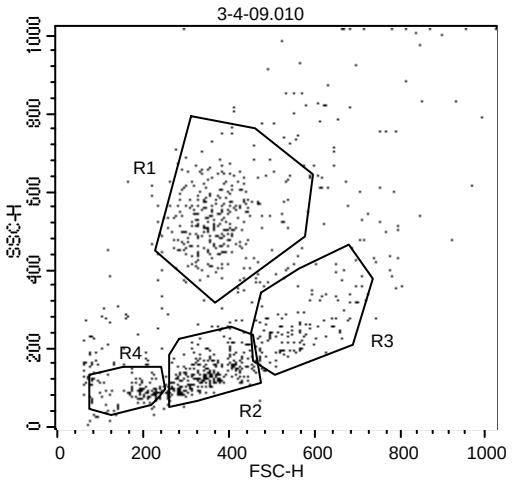
Cells in Region 2 and 3 followed a similar pattern in CD11a/CD18 expression, as it was discussed previously for CD44 with fresh and two hour non-adherent cells

Figure 3.3 Flow cytometric analyses of cell surface molecule expression in freshly isolated bone marrow cells compared to cells cultured 2 hours. A-C) Dot plot distribution of bone marrow cells isolated using gradient density centrifugation followed by either A) fresh/uncultured analysis or analysis of the B) non-adherent or C) adherent cell fractions after two hours of culture. Note that the size, granularity, and distribution of the adherent cell population following two hours of culture are different from freshly isolated and non-adherent cells. After two hours of culture, cells in Region 2 were primarily non-adherent, while cells in Region 3 were primarily the adherent type. D) Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated areas (Regions 1, 2, and 3 respectively). The shaded curves represent negative isotype control staining; open lines represent the labeling for the cell surface markers indicated in the left-hand side. No notable differences were detected in molecule expression in Region 1 in all markers analyzed. There appear to be only minor differences between freshly isolated cells and cells that were non-adherent after two hours of culture. Note the dramatic difference in cell surface expression in Regions 2 and 3 for all CD markers analyzed between freshly isolated or 2 hour non-adherent cells compared with cells which were adherent after 2 hours of culture. Short term culture of two hours can be used to separate cells with varying maturities based on adherence. Cells which are non-adherent at two hours of culture appear to be similar in expression profile to freshly isolated bone marrow cells, while adherent cells possess a different expression phenotype consistent with more mature cells.

A. Fresh/uncultured



B. 2 hour non-adhered



C. 2 hour adhered

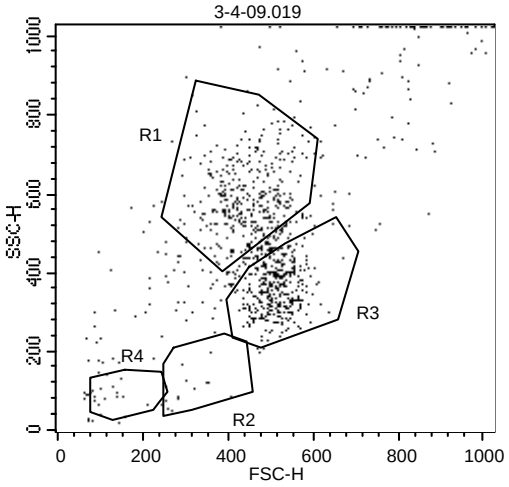
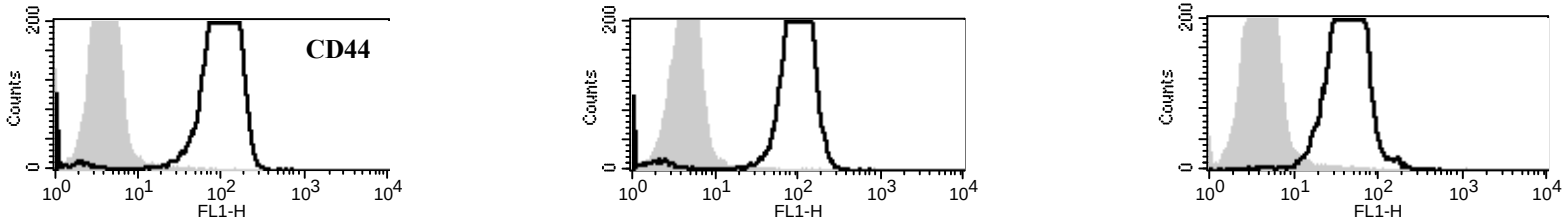
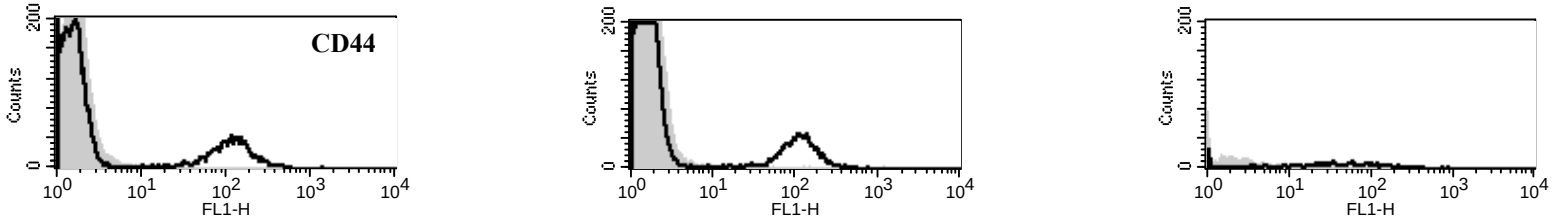


Figure 3.3 (Continued)

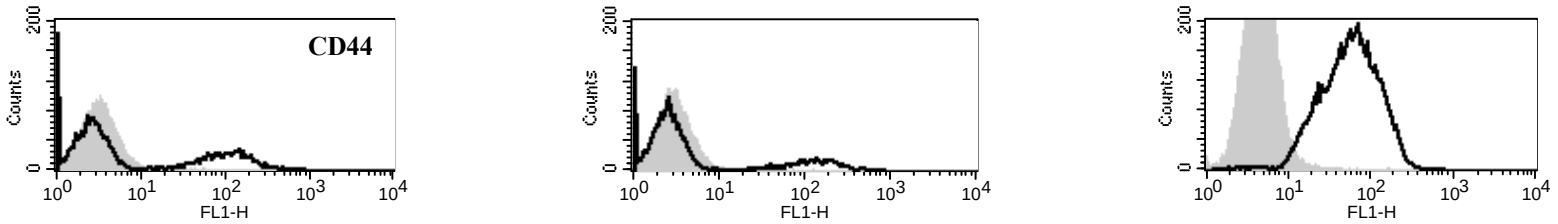
D. **Fresh** **2 hour non-adhered** **2 hour adhered**



Region 1 (size/granularity equivalent to neutrophils)



Region 2 (size/granularity equivalent to lymphocytes)



Region 3 (size/granularity equivalent to monocytes)

Figure 3.3 (Continued)

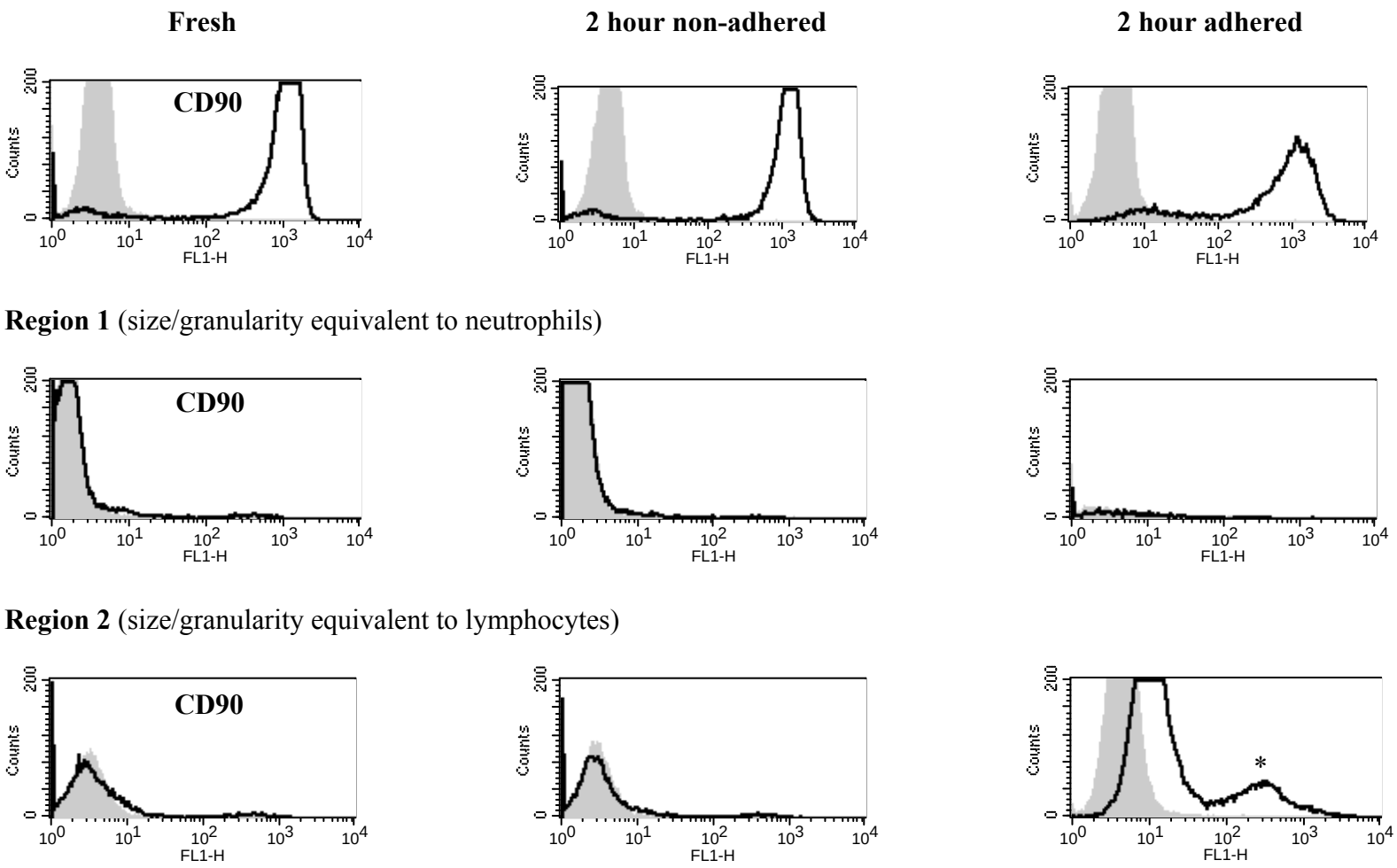
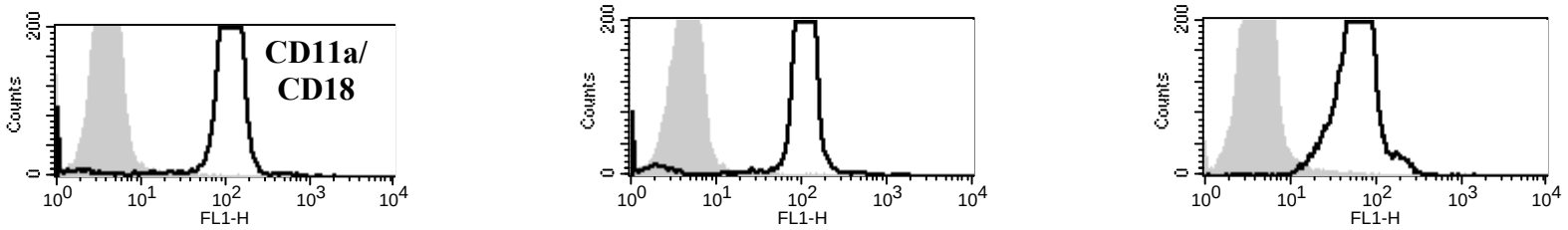
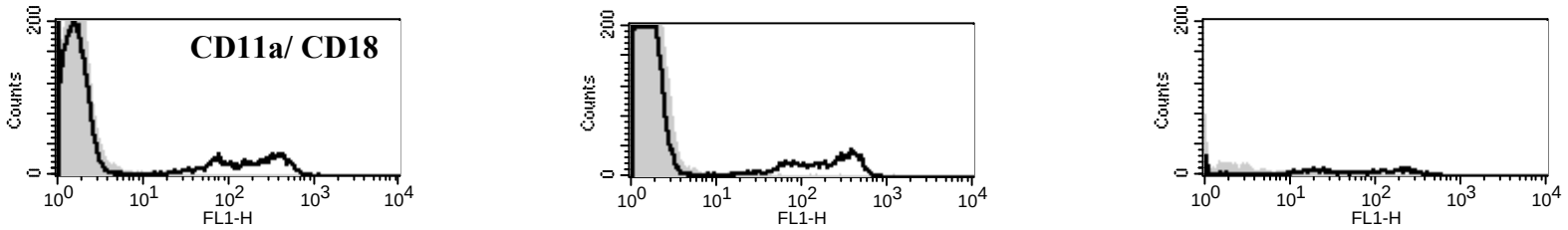


Figure 3.3 (Continued)

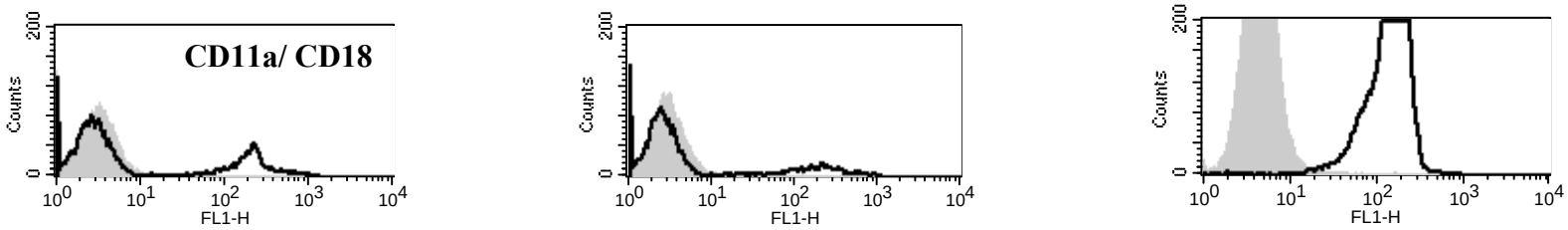
Fresh **2 hour non-adhered** **2 hour adhered**



Region 1 (size/granularity equivalent to neutrophils)



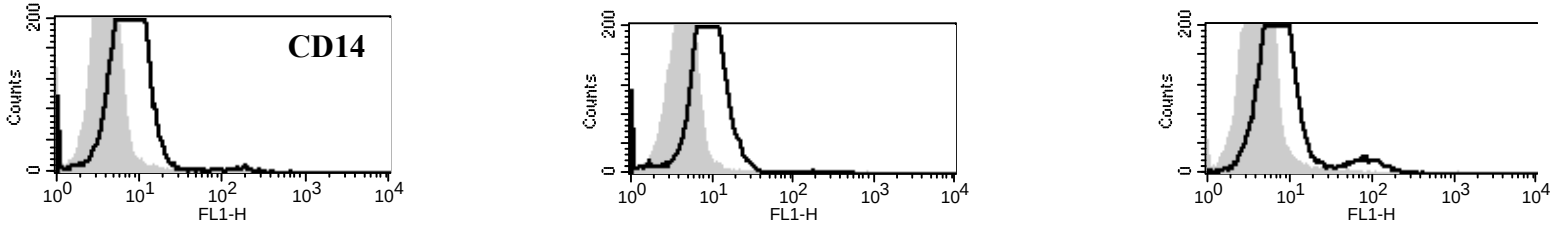
Region 2 (size/granularity equivalent to lymphocytes)



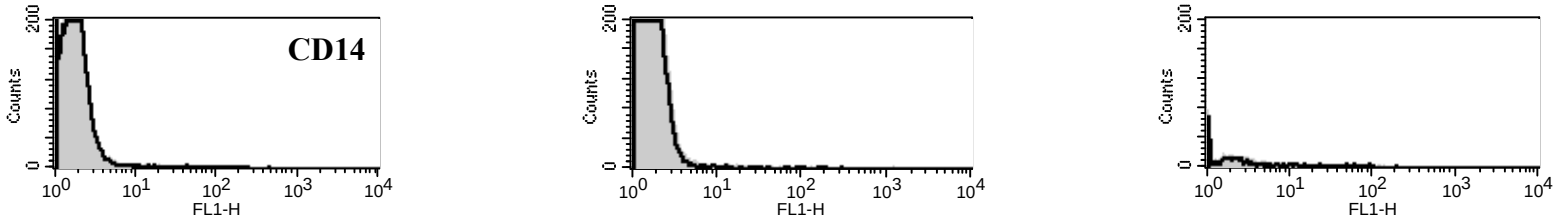
Region 3 (size/ granularity equivalent to monocytes)

Figure 3.3 (Continued)

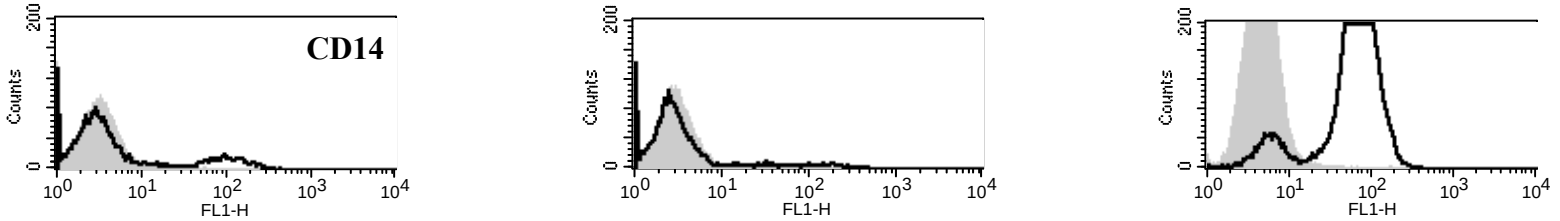
Fresh **2 hour non-adhered** **2 hour adhered**



Region 1 (size/granularity equivalent to neutrophils)

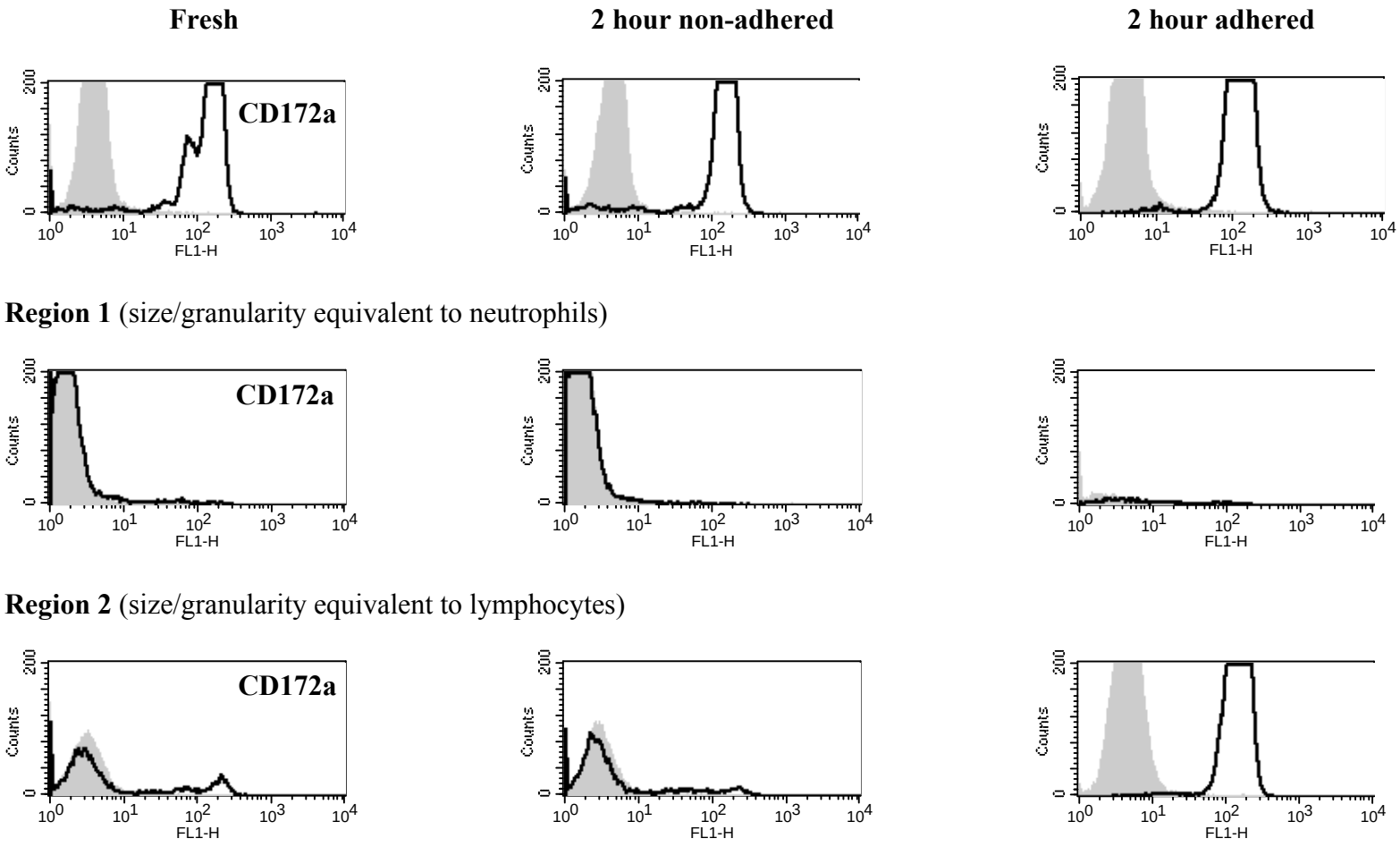


Region 2 (size/granularity equivalent to lymphocytes)



Region 3 (size/ granularity equivalent to monocytes)

Figure 3.3 (Continued)



having a low number of positive cells while the adherent cells in these regions were nearly all brightly positive for CD11a/CD18 expression. CD14 expression was low in mean fluorescence intensity for Region 1 in all three fractions, consistent with the pattern seen in neutrophils. In Region 1, a small population of CD14 brightly positive cells was noted, again consistent with overlapping gating between neutrophil and monocyte populations. Cells in Region 2 were negative for CD14 expression, consistent with the expression pattern of lymphocytes. Cells in Region 3 from the adherent fraction were primarily brightly positive with a smaller population of low positive cells, consistent with the pattern that primarily monocytes were present with a few neutrophils also present. CD172a expression once again was positive for all three groups in Region 1. Cells in Region 2 were negative for CD172a expression, again consistent with the expression profile of lymphocytes. Cells in Region 3 had very small populations of CD172a positive cells in fresh and non-adherent cell fractions while adherent cells were uniformly brightly positive.

Flow cytometric analysis of cell surface marker expression in cultured bone marrow cells: After two days of culture, adherent mononuclear cells displayed an antibody labeling pattern of $CD44^{hi}$, $CD90^{hi}$, $CD11a/CD18^{mod}$, $CD14^{mod}$, and $CD172a^{high}$ (**Figure 3.4**). In Gate 3, there appeared to be a heterogeneous cell population as evidenced by multiple peaks in CD44, CD90, and CD14 mean fluorescence intensity. On day seven, CD44 expression decreased slightly in mean fluorescence intensity, but remained strongly positive and became more homogenous. CD90 expression was negative in two of the three cell populations present, but high mean fluorescence intensity was noted in a third population. Meanwhile, CD11a/CD18, CD14, and CD172a cell surface expression decreased, with a shift in the populations to lower mean fluorescence intensity. By fourteen days, adherent cells were $CD44^{hi}$, $CD90^{hi/variable}$, $CD14^{low}$, $CD172a^{low}$ and $CD11a/CD18^{neg}$; these cells displayed a

Figure 3.4 Flow cytometric analyses of cell surface molecule expression in cultured bone marrow cells from two through twenty-one days of culture. **Part A-D** dot plot distribution of bone marrow cells cultured A, 2 days ; B, 7 days ; C 14 days ; or D 21 days. **Part E** Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated areas (Regions 1, 2, and 3 respectively). The shaded curves represent negative isotype control staining; open lines represent the labeling for the cell surface markers indicated in the left-hand side. Note that samples at 2 and 7 days of culture have multiple populations present indicated by multiple peaks in the overlay histograms. By day 14, the cell population is more homogeneous in mean fluorescence intensity for most markers, but remained variable for CD90 protein expression. At 21 days of culture, putative MPCs were $CD44^{hi}$, $CD90^{hi}$, $CD14^{low}$, $CD172a^{low}$ and $CD11a/CD18^{neg}$ with a homogenous population in all markers analyzed. The mean fluorescence intensity of CD14 expression is reduced over time in later samples compared to mean fluorescence intensity on day 2, but continues at low levels throughout culture.

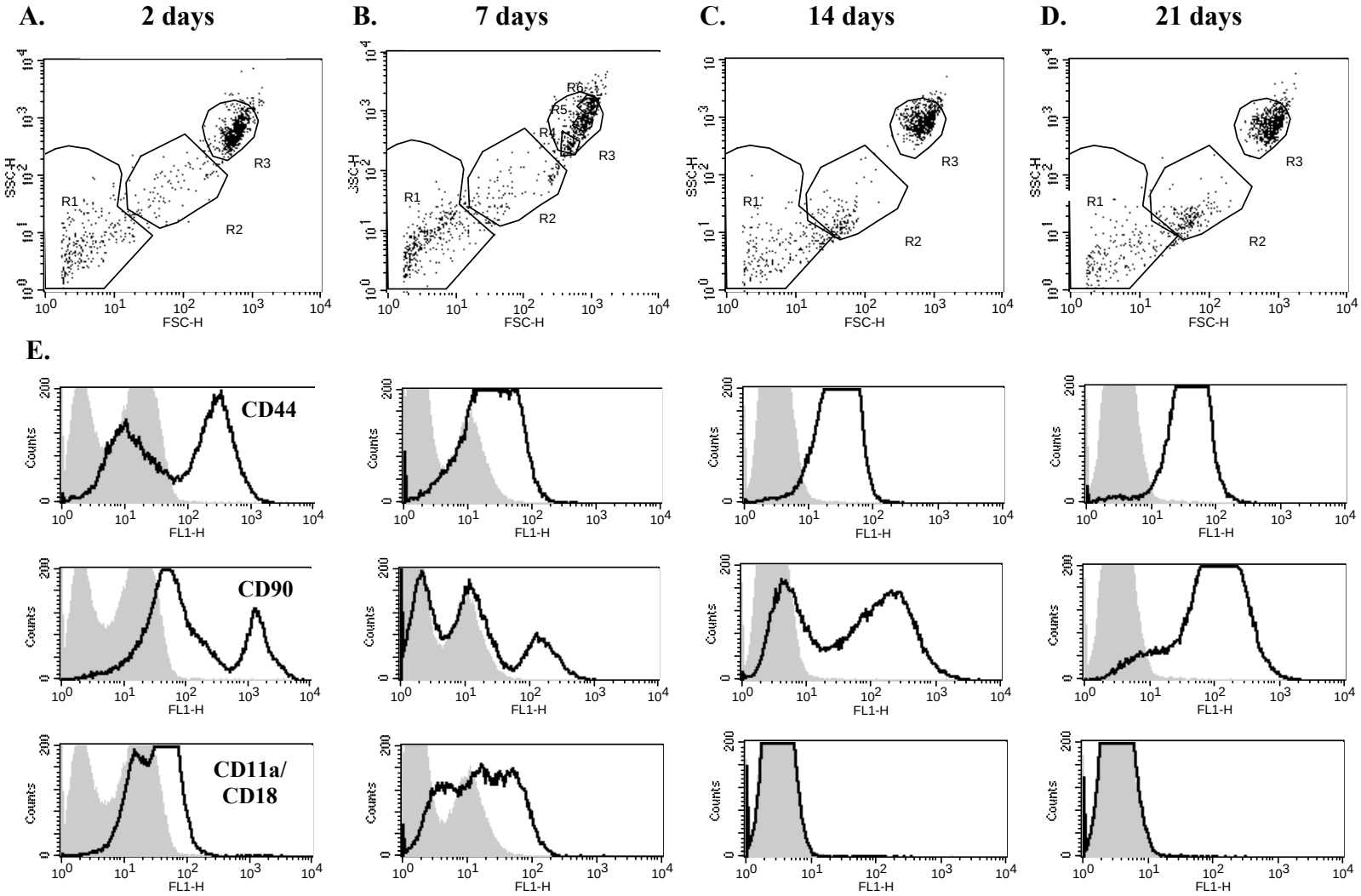
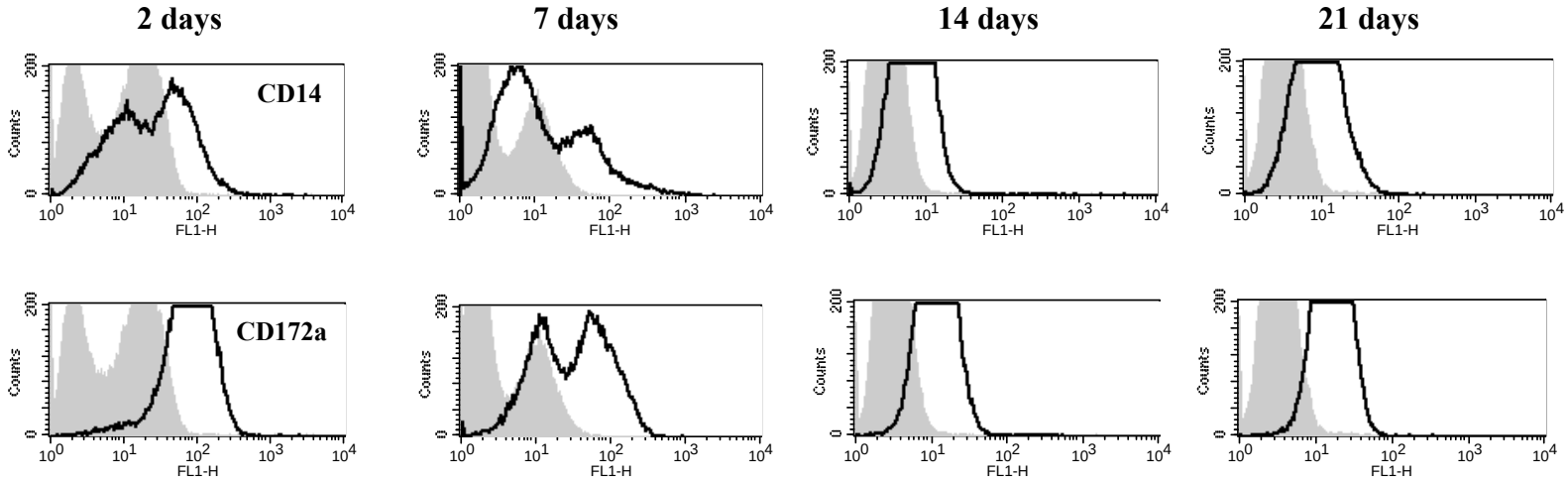

A.
2 days
B.
7 days
C.
14 days
D.
21 days
R1
R2
R3
R4
R5
R6
SSC-H
FSC-H
E.
CD44
CD90
CD11a/
CD18
Counts
FL1-H

Figure 3.4 (Continued)



fibroblastic morphology characteristic of MPC (data not shown). Multiple cell populations were noted via several peaks in CD90 mean fluorescence intensity at 14 days, with a growing number of positive cells compared to percentages at 7 days. All other markers had homogeneous protein expression by 14 days. This pattern of molecule expression was retained at 21 days for all MPC samples, with CD90 becoming homogenous with high mean fluorescence intensity. At all time points (2, 7, 14, and 21 days), adherent bone marrow cells in culture showed a consistent protein phenotype. The only variable between horse samples was the number of days (14 or 21) needed before the predominant cell population was homogeneous for CD90 expression.

Gene expression kinetics of CD14 in cultured bone marrow cells over time: *CD14* gene expression data was consistent with cell surface protein expression at all culture time points. *CD14* gene expression was present in all samples but became significantly less ($P \leq 0.005$) over time (**Figure 3.5**). Samples cultured one week or less had significantly more *CD14* expression than samples cultured more than one week. CD14 expression remains stable at low levels after 14 or more days of culture with less than one fold change compared to the control sample.

Magnetic activated cell sorting using the mouse anti-horse CD14 antibody: Cell sorting of the adherent cells following two days of culture led to distinct distributions of cells within the positive and negative fractions compared to unsorted conditions following MACS (**Figure 3.6**) as assessed by flow cytometry. The CD14 positive fraction primarily concentrated cells in Region 3, while CD14 negative selection primarily concentrated cells in Region 2.

Quantification of colony formation: Following a week of culture, a significant difference in MPC colony formation was noted between all three fractions ($P \leq 0.005$) (**Figure 3.7**). Cells positively selected for CD14 expression had significantly more

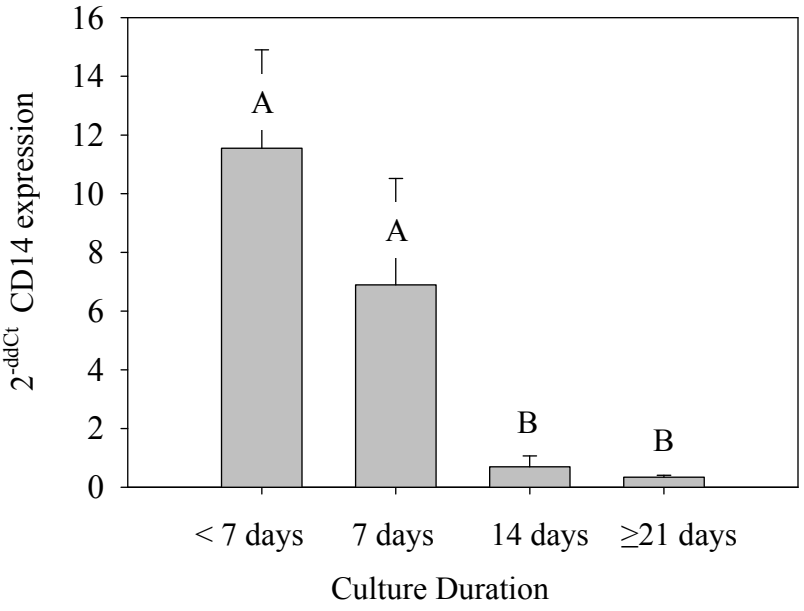


Figure 3.5 Gene expression of *CD14* during increasing culture durations. *CD14* expression was significantly higher in cells cultured one week or less compared to cells cultured more than one week ($P \leq 0.005$). Despite a significant decrease over time, *CD14* expression was detected in all samples. A bone marrow sample cultured for 14 days was used as the control sample for comparison using the 2^{-ddCt} method. Bars represent n=6 ± SE, letters denote significant differences when analyzed using one-way ANOVA and Tukey's All-Pairwise Comparisons.

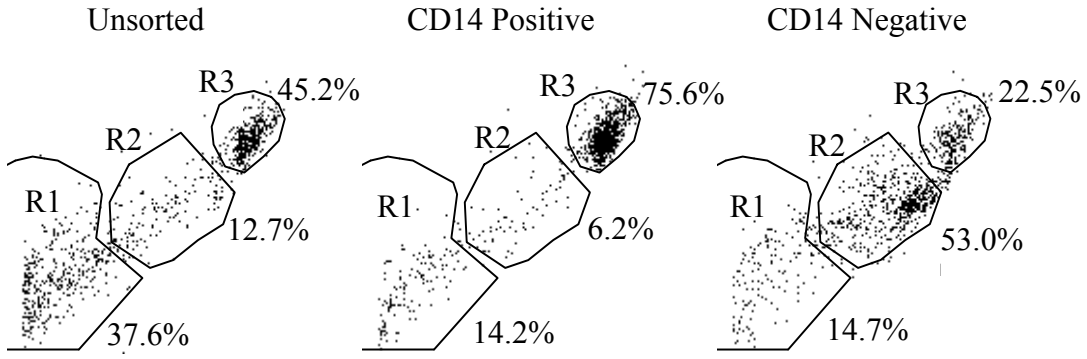


Figure 3.6 Flow cytometric dot plot analysis of equine bone marrow cells cultured for 2 days, following MACS sorting using a mouse anti-equine CD14 antibody. Note that the percentage distribution of cells in each region varies between fractions.

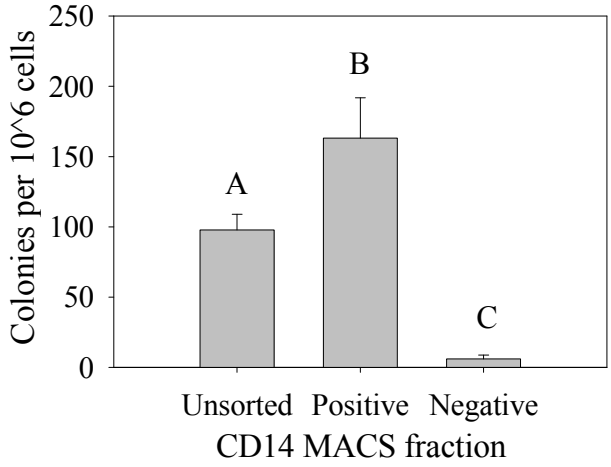


Figure 3.7 Colony counts on day 7 of culture in bone marrow cells with or without selection using an anti-equine CD14 antibody at 2 days of culture. The number of MPC colonies formed per 10^6 cells in each fraction were tested by one-way ANOVA, blocked by horse, and Tukey's All-Pairwise Comparisons post hoc (n=6±SE). There were significant differences ($P \leq 0.005$) between all three groups. Positive selection using an anti-equine CD14 antibody appears to enrich the putative MPC population over negative selection and unsorted conditions.

MPC colonies formed than either unsorted or negatively selected fractions, with nearly double the colony counts of unsorted cells and over twenty times the number of colonies formed in the CD14 negatively selected fractions.

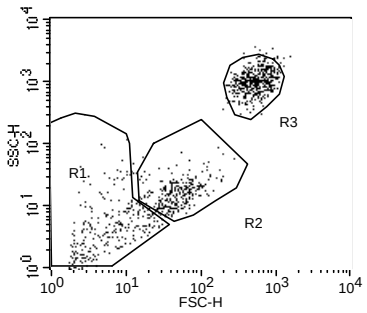
CD14 gene expression over time between sorted and unsorted cells: There were no significant differences between *CD14* expression in any of the three fractions at 14 (n=3; p=0.013), 21 (n=6; p=0.87), or 30 (n=6; p=0.49) days of culture. Many horses (n=3) did not have sufficient MPC cell numbers (≥ 50,000) in the negatively selected fraction at fourteen days for analysis. The small sample size (n=3) at 14 days may have prevented detection of a significant difference between fractions. An unsorted bone marrow sample which had been cultured for 14 days was used as the control sample for comparison using the 2^{-ddCt} method. Statistical analysis was performed using one-way ANOVA and Tukey's All-Pairwise Comparisons.

Flow cytometric analysis of CD14 expression in cultured MPCs in response to LPS stimulation: There were no detectable differences in CD14 mean fluorescence intensity present between unsorted and CD14 positively selected MPCs after 21 days of culture, with or without overnight LPS stimulation as assessed using flow cytometry (data not shown). There was a detectable difference in mean fluorescence intensity between untreated and LPS stimulated cells in both cell fractions (**Figure 3.8**). All doses of LPS (1, 5, or 10 ng/mL media) caused an increase in both percentage of positive cells and CD14 mean fluorescence intensity, suggesting equine MPCs respond to LPS stimulation with an upregulation of CD14 protein expression.

RT-qPCR analysis of *CD14* gene expression in cultured MPCs in response to LPS stimulation: There were no detectable differences in CD14 expression between unsorted and positively selected MPCs following 21 days of culture as assessed using RT-qPCR. Both fractions had similar levels of *CD14* expression to control samples, and displayed a similar increase in *CD14* expression in response to LPS stimulation

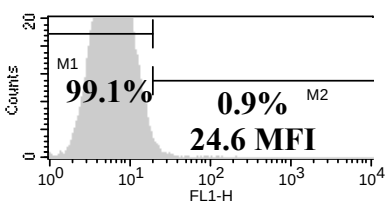
Figure 3.8 Flow cytometric analysis of CD14 cell surface molecule expression in unsorted bone marrow cells in response to LPS stimulation. Note that cells in Region 3 appear to be responsive to LPS stimulation, increasing both percentage of positive cells and mean fluorescent intensity over control conditions. **Part A**. Dot plot distribution of unsorted bone marrow cells cultured 21 days. **Part B**. Histogram analysis of CD14 mean fluorescence intensity in Region 3 following overnight incubation of cells with LPS (026:B6, L2762; Sigma-Aldrich, Saint Louis, MO) of differing concentrations (0, 1, 5, or 10 ng/mL media). The histograms represent cell surface molecule expression using either a negative isotype control antibody (left column) or the mouse anti-equine CD14 antibody with FITC conjugated IgG secondary antibody (right column). M1 represents the setting used for negative cell percentage calculations based on isotype control labeling. M2 represents the setting for positive cell percentage calculations. (LPS, lipopolysaccharide; MFI, mean fluorescence intensity)

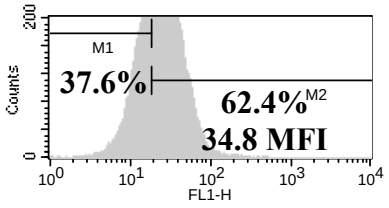
A. Unsorted



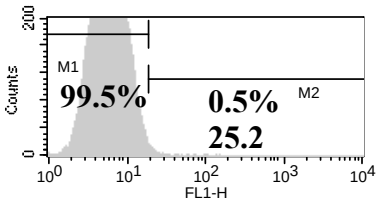
B.

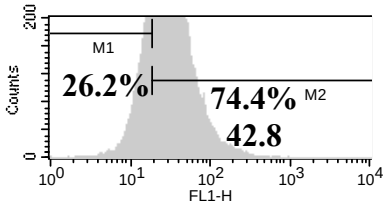
No LPS added **Negative control** **CD14**



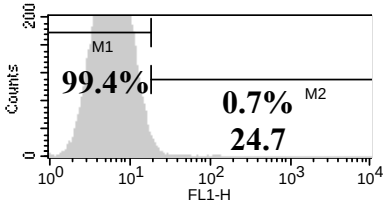


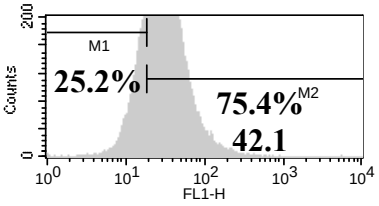
LPS 1 ng/mL



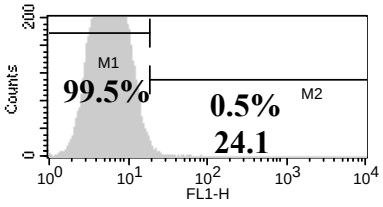


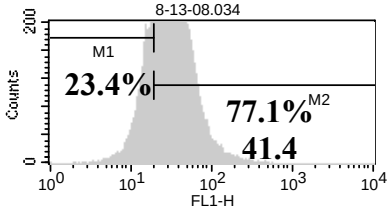
LPS 5 ng/mL





LPS 10 ng/mL

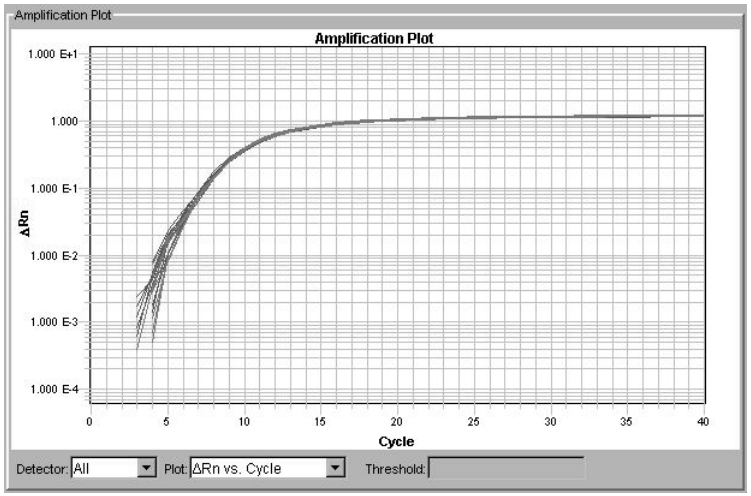




(**Figure 3.9**). Analysis of 2^{-ddCt} demonstrates a significant difference in *CD14* expression between untreated and LPS stimulated cells in both fractions ($p \leq 0.005$). culture time point (e.g. 2 days of culture) in order to determine if the CD11a/CD18 epitope is trypsin labile. The low mean fluorescence intensity of CD14 expression. Cells treated with any dose of LPS (1, 5, or 10 ng/mL media) had a 2.24 (±0.12) fold increase in CD14 expression over untreated cells (**Figure 3.9**). Both unsorted and CD14 positive selected MPCs respond to LPS stimulation with an up regulation of CD14 expression, supporting the flow cytometry data.

Analysis of cell surface marker expression in response to trypsinization: Flow cytometric data demonstrated no detectable difference between sorted and unsorted fractions in cells cultured 30 days for all cell surface molecules tested (**Figure 3.10**). MPCs in all fractions (unsorted, CD14 positive, or CD14 negatively selected) were $CD44^{hi}$, $CD90^{hi}$, $CD11a/CD18^{neg}$, $CD14^{low}$, and $CD172a^{low}$ in phenotype, similar to initial bone marrow flow cytometry results described previously when cells were harvested using Accumax® cell dissociation solution. Following harvest using 0.25% trypsin in HBSS, there was a change in phenotype in all fractions to $CD44^{low}$, $CD90^{hi}$, $CD11a/CD18^{neg}$, $CD14^{neg}$, and $CD172a^{low}$. Statistical analysis of flow cytometry data demonstrates significant drops ($P \leq 0.005$) in percentages of positive cells following cell harvest using trypsin versus Accumax® cell dissociation solution (**Figure 3.11)** in several protein markers. The mean fluorescence intensity for CD44 was decreased in all samples when trypsin was used for cell harvest, but expression was still present. The percentage of CD44 positive cells dropped from 98.05% (±4.09) to 39.98% (±4.56) when trypsin was used for cell harvest. There appears to be no effect on CD90 mean fluorescence intensity when trypsin in used for cell harvest, with no difference in the percentages of positive cells between treatments. CD11a/CD18 had a

A.



B.

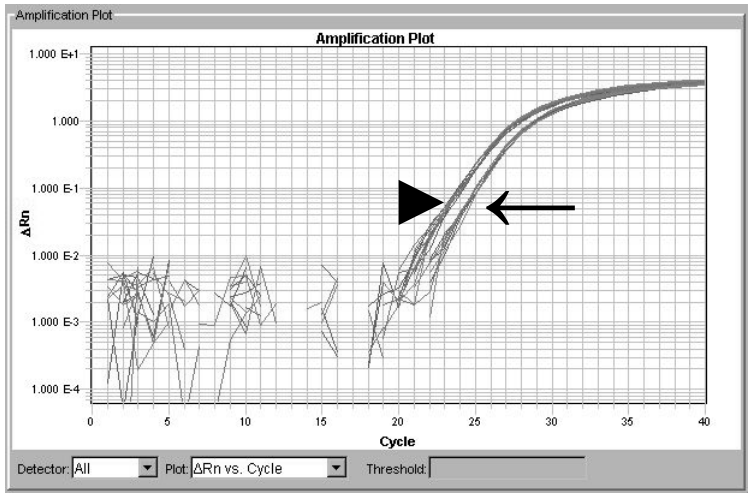


Figure 3. 9 Gene expression kinetics of *CD14* in equine bone marrow cells which were unsorted or CD14 positive selected (at two days of culture). MPCs were subsequently cultured 21 days and treated with LPS (026:B6, L2762; Sigma-Aldrich, Saint Louis, MO) at dosages of 0, 1, 5, or 10 ng/mL media. **Part A** *18S* gene amplification plot verifies equal loading of RNA between control and treated samples. **Part B** *CD14* amplification plot of LPS stimulated and control bone marrow cells. Putative MPCs treated with 0 ng/mL of LPS (←) had higher Ct values (therefore lower levels of *CD14* expression) than cells which were treated with LPS in any dose (►). By 21 days there is no detectable difference in *CD14* expression between the CD14 positive selected and unsorted cells within the control and LPS stimulated groups. Both groups respond to LPS stimulation with an upregulation of *CD14* expression, supporting the previous flow cytometry data. Analysis of 2^{-ddCt} demonstrates a significant difference ($P \leq 0.005$) with LPS treated cells showing a 2.24 (±0.12) fold increase in *CD14* expression over untreated cells. (LPS, lipopolysaccharide)

Figure 3.10 Flow cytometric analysis of cell surface molecule expression in sorted and unsorted bone marrow cells cultured 30 days. **Parts A & B**. Dot plot distribution of bone marrow cells from the respective fractions following MACS sorting using an anti-equine CD14 antibody at 48 hours of culture, and subsequent culture until 30 days. Adherent cells were collected for analysis following five minutes of incubation at 37°C with either Accumax® cell detachment solution (**Part A**) or 0.25% trypsin in HBSS (**Part B**). **Part C.** Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated area (Region 3). Shaded curves represent negative isotype control staining; open lines represent labeling for the cell surface markers indicated on the left-hand side. Note the similarity in protein expression between the unsorted, positive, and negative fractions when Accumax® cell detachment solution is used, suggesting the cells in each fraction are phenotypically similar at the 30 day time point. When trypsin is used to collect the cells for analysis, note the decrease in mean fluorescence intensity for CD44 and CD172a and the absence of mean fluorescence intensity for the CD14 sample compared to the isotype control, suggesting these epitopes are trypsin labile. The positive and negative sorted fractions showed a similar pattern of decrease in CD44, CD172a and CD14 protein expression when cells were collected using trypsin (data not shown) compared to the unsorted fraction shown in Part C (rightmost column). CD90 did not change in mean fluorescence intensity and did not appear to be affected by trypsin. The effect of trypsin on CD11a/CD18 expression could not be determined using a 30 day cultured sample since protein expression levels of this marker were low in all groups.

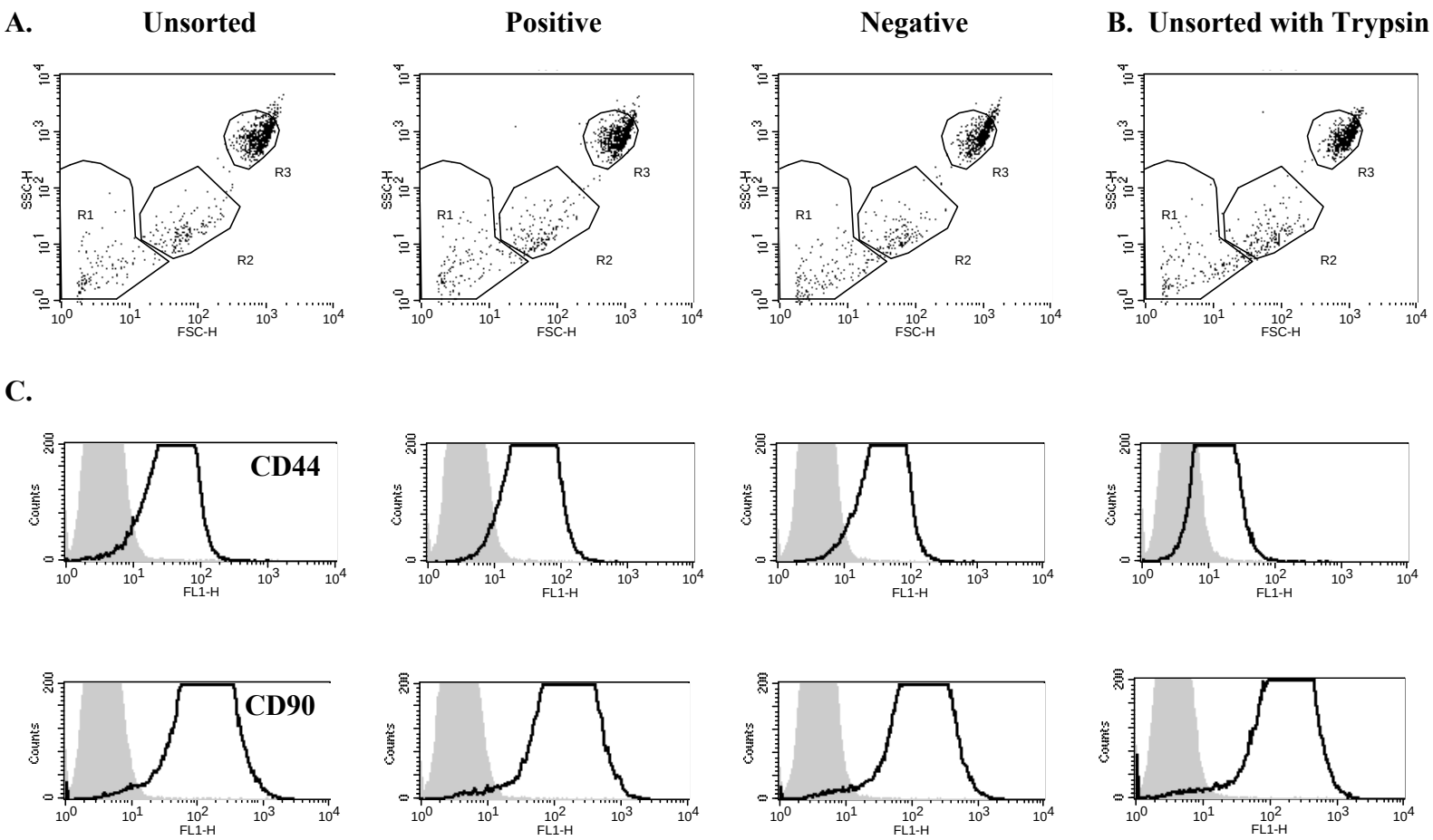
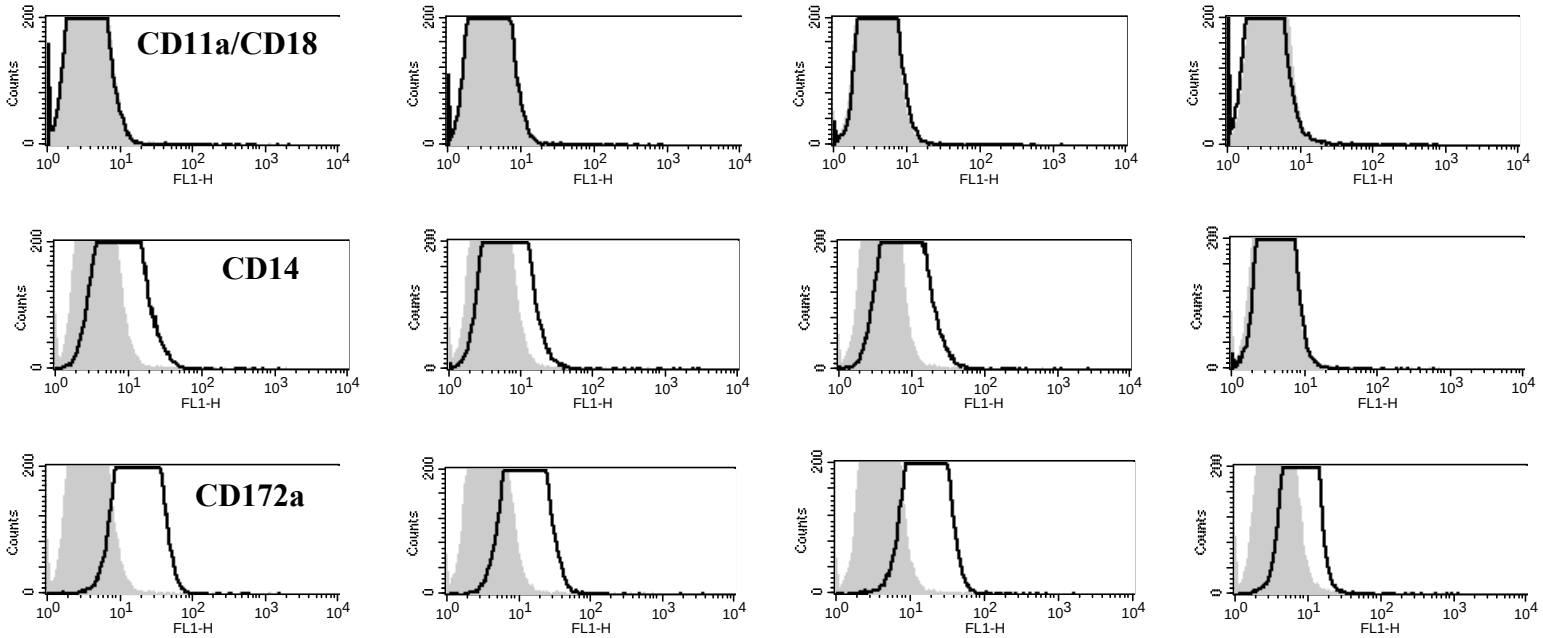

A.
Unsorted
Positive
Negative
B. Unsorted with Trypsin
R1
R2
R3
SSC-H
FSC-H
C.
CD44
CD90
Counts
FL1-H

Figure 3.10 (Continued)



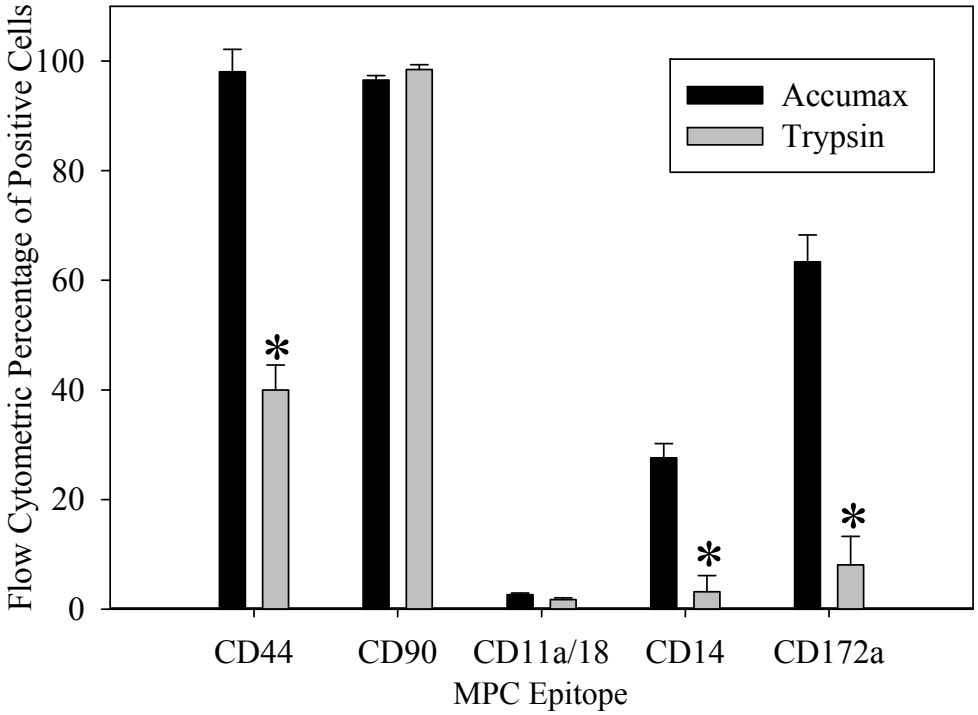


Figure 3.11 Flow cytometric analysis of cell surface molecule expression in bone marrow cells cultured 30 days and lifted following 5 minutes of incubation at 37°C with either Accumax® or 0.25% Trypsin in HBSS. The percentage of positive cells using the respective antibody was compared between solutions, and tested by One-Way ANOVA, blocked by horse, and Tukey's All-Pairwise Comparisons post hoc (n=4 ± SE). There were significant differences ($P \leq 0.005$) in positive percentages of CD44, CD14, and CD172a expression (*) when trypsin was used instead of Accumax® for cell preparation. There was no significant difference in positive percentage of CD90 expression between cell lifting solutions. The overall percentage of positive cells using CD11/CD18 antibody was very low in both treatment groups, making comparison between solutions difficult at this time point in culture (no significant difference was detected). Lifting cells with a 0.25% Trypsin solution for flow cytometric analysis reduces the detection of some but not all cell surface markers.

trend (P=0.06) toward decreased percentages of positive cells, but the low percentages of positive cells in both treatments do not allow for any conclusions to be drawn on the effect of trypsinization. Bone marrow cells would need to be tested at an earlier time point to determine if the CD11a/CD18 epitope is trypsin labile. The low mean fluorescence intensity of CD14 disappeared following trypsinization compared to cells that were harvested using Accumax®, suggesting that the CD14 epitope is trypsin labile. The percentage of CD14 positive cells was significantly reduced from 27.60% (±2.61) to 3.18% (±2.92) following incubation with trypsin, causing the trypsin treated samples to appear negative for CD14 expression, suggesting that the CD14 epitope is trypsin labile. A decrease in mean fluorescence intensity for CD172a was also noted in all fractions when trypsin was used for cell harvest. There was a significant drop in CD172a percentages from 63.39% (±4.89) to 8.08% (±5.18) when trypsin was used for cell harvest. Harvesting cells for flow cytometry analysis with trypsin appears to damage several cell surface proteins, while others appear to be unaffected. *CD14* gene expression analysis of cells used in the comparison between trypsin and Accumax® cell harvest solutions demonstrated a low level of *CD14* gene was present in all samples at levels (data not shown) comparable to 30 day cultured MPC data presented above.

Discussion

In this study, temporal changes in protein and gene expression of the cell surface marker CD14 in bone marrow cells were demonstrated. Comparison of freshly isolated bone marrow cells with cells that were primarily adherent or non-adherent after two hours of culture showed distinct differences in CD14 and other cell surface marker proteins between fractions. The mean fluorescent intensity of CD14 expression and the percentages of CD14 positive cells were much higher in the

adherent monocyte-sized population when compared to cells that were monocyte-sized from the freshly isolated or non-adherent fractions (**Figure 3.3,** R3).

Most adherent cells following two hours of culture would be expected to be granulocyte (**Figure 3.3**, R1) or monocyte lineages (**Figure 3.3**, R3), with few lymphocytes (**Figure 3.3**, R2) present. Culture of two hours duration appears to be useful in separating bone marrow cells types with varying properties based on adherence. Cells which were non-adherent at two hours of culture appear to be similar in expression profile to freshly isolated cells, while adherent cells possess a different expression phenotype, consistent with adherent hematopoietic cells. These results suggest that separation of freshly isolated bone marrow cells may lead to inaccurate classification of hematopoietic versus non-hematopoietic cells. Freshly isolated hematopoietic cells may not express cell surface proteins (e.g. CD11a/CD18 or CD14) normally associated with their hematopoietic lineage, leading to contamination of the true non-hematopoietic population with hematopoietic cells. Caution should be exercised when evaluating results of studies that attempt to sort hematopoietic from non-hematopoietic cells using uncultured bone marrow samples.

One of the defining features of MPCs in humans and other species is lack of expression of the CD14 epitope, also known as the lipopolysaccharide receptor (LPS-R) on their cell surface. However, I have demonstrated that CD14 is expressed on the surface of putative equine MPCs and can be used as a marker to enrich for the MPC population. Mean fluorescence intensity and *CD14* gene expression decrease significantly in established MPC populations compared to cells cultured seven days or less; however after 14 days of culture, CD14 expression at both the protein and gene level was stable. This is in contrast to cell surface markers such as CD11a/CD18, which were negative in equine MPCs cultured 14 or more days. All RNA samples analyzed by RT-qPCR were able to reach a Ct value for *CD14* expression, unlike

CD11a and *CD45* in a previous study [20], suggesting that *CD14* expression is present in equine MPCs.

MACS using an anti-equine CD14 antibody was successful in enriching MPC colony formation in the CD14 positive fraction compared to unsorted and negatively selected fractions. The procedure was relatively easy to perform; however, several factors are required to use this technique successfully. You must choose an epitope which is present in sufficient quantities on the cell surface of the positive population (so they will be retained in the column), but not the negative population (so they will flow-through the column) or you will not concentrate the desired cell type. A primary antibody that can be strongly bound by the secondary microbeads is also essential for successful MACS. Care is needed to avoid overloading the column with excessive numbers of cells, otherwise positive cells will leak into the negative fraction. Not every antibody works well in the MACS application and I was fortunate to have the anti-equine CD14 antibody provided. Preliminary work using the BiG10 anti-human CD14 antibody for MACS of equine bone marrow cells led to similar overall results with many more MPC colonies in the positive versus negative fractions, with a distinct separation of approximately ten times (versus over twenty times using the anti-equine antibody) in the number of colonies in the positive fraction compared to the negative fraction (data not shown).

Flow cytometry was useful in the assessment of purity of sorted cells using MACS. Previous work from our laboratory [20] suggests that MPCs come from the region concentrated in the CD14 positive fraction (R3) as shown in **Figure 3.6**. The percentage of positively selected cells located in this region was nearly double the percentage of unsorted cells, and 3-5 times more than the negatively selected cell fraction. Comparison of percentages in cell distribution between cell fractions helps confirm an initial difference between the unsorted and sorted fractions.

Contrary to my hypothesis, the MPC population was not enriched in the CD14 negative fraction of equine bone marrow cells. Putative MPC colonies were enriched in the CD14 positive fraction approximately two-fold over unsorted, and more than twenty-fold over negatively selected cells at day seven of culture (**Figure 3.7**). These results suggest that equine MPCs are not CD14 negative as reported in other species.

CD14 gene expression between unsorted and sorted cells was not different in cells cultured 14 days or more. However, half of the samples from the MACS sorting experiment (n=3) did not have sufficient MPC cell numbers in the negatively selected fraction to rule out the possibility that a difference in *CD14* expression exists. More sorted samples cultured 14 days will need to be prepared to confirm that the lack of a significant difference in CD14 expression is real. By 21 days, MPCs from each fraction appear to have equivalent *CD14* expression, suggesting that the MPCs growing in all three fractions are the same cells. The difference between fractions appears to be the quantity of MPCs available for analysis at early time points (e.g. at 14 days, few MPCs were present in the negative fraction in all samples; only half of the negative samples had ≥ 50,000 cells, sufficient for RNA extraction). RNA samples from the 48 hour MACS separation are still awaiting analysis and will answer the questions whether an initial difference in *CD14* expression exist between the fractions. If a difference in *CD14* expression is detected, it will provide additional supporting evidence of purity of MACS sorted cells.

CD14 protein and gene expression were both upregulated in equine MPCs following stimulation by LPS. Cells outside of the hematopoietic lineage should not be capable of responding to LPS stimulation when isolated from cells that normally express CD14 on their cell surface (e.g. myeloid cells such as monocytes, dendritic cells, and neutrophils). Therefore, either hematopoietic cells, such as monocytes and dendritic cells, are still present in MPC cultures at 21 days (stimulating CD14

expression in MPCs through secretion of the soluble form of CD14) or cultured MPCs do have low levels of CD14 expression on their cell surface and are directly responding to LPS. If the latter is true, the non-hematopoietic lineage of equine MPCs must be questioned.

In humans and other species, MPCs are reported to be negative for CD14 protein expression as assessed by flow cytometry. However, many studies consider CD14 negative, even though they report low levels of CD14 expression. For example, a recent MPC characterization study performed in sheep reported 28.5% (±13.8) of cultured MPCs were positive for CD14 expression at passages 3 and 4, but still classified the ovine cells as CD14 negative [21]. The percentage of positive ovine MPCs in that study may have also been artificially lowered since trypsin was used for cell harvest prior to flow cytometric analysis.

In the original characterization paper by Pittenger et al., CD14 expression was reported as negative in human MPCs [6]. In that study, cells were harvested using either trypsin or EDTA, with no reported difference in cell surface protein detection for any marker between cell dissociation solutions. Most subsequent MPC characterization studies have used the protocol described by Pittenger, including trypsinization, for cell harvest prior to flow cytometry analysis. I have clearly demonstrated that equine MPCs had a low mean fluorescence intensity of CD14 detection when cells were lifted with Accumax® cell dissociation solution. When trypsin was used instead, CD14 expression was no longer detected in samples from the same MPC fractions. My data suggest that CD14 is a trypsin labile protein in the horse. Some cell surface proteins were also found to be trypsin labile including CD44 and CD172a, while others such as CD90 had no detectable sensitivity to trypsin. Given these findings, the use of trypsin prior to flow cytometry analysis in equine bone marrow cells is not recommended.

My results suggest that equine MPCs are enriched by selection for CD14 protein expression. Equine MPCs also appear to have long term expression of CD14 protein in cultured cells, which can be upregulated by stimulation with LPS, and damaged by exposure to trypsin, making them appear falsely negative for CD14. A plausible conclusion from these findings is that equine MPCs are derived from a CD14 positive precursor cell. Cells of stromal origin should not be CD14 positive, suggesting that equine putative MPCs do not fit the typical classification of stromal cells. My data supports the premise that equine MPCs are more likely derived from a hematopoietic precursor. In the future, I hope to further evaluate the potential role of MPCs in tissue regenerative therapeutic and research applications.

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CHAPTER 4

SUMMARY AND CONCLUSIONS BASED ON EXPERIMENTAL RESULTS

What is the True Lineage of Mesenchymal Progenitors in the Horse?

Classically, MPCs have been defined and characterized as non-hematopoietic in origin. The reasons behind this assumption include the highly proliferative behavior of these cells in culture, their response to *in vitro* "differentiation", and their lack of expression of certain cell surface proteins thought to be characteristic of hematopoietic cells. At this time, my results question the assertions that MPCs represent "true" mesenchymal stem cells and that they arise from a non-hematopoietic origin.

As much as MPCs differ from hematopoietic cells in certain cell surface characteristics, they also do not share many properties of "stem cells". For example, MPCs cannot self renew long term, and typically can be culture expanded for only a few weeks before their proliferation slows. Rat MPCs cultures have been shown to undergo senescence during the first 30 days in culture, with an increase in population doubling time, decreased DNA repair, and loss of telomerase activity [1]. It has been proposed that human MPCs enter senescence as soon as they are placed into culture [2]. The average population doubling of human MPCs derived from bone marrow is reported as 38±4 times [3], which means the total lifespan of these cells in culture is limited to a timeframe of a few months. A true stem cell divides infrequently and should be able to self renew for the lifetime of the organism, so clearly cultured MPCs do not meet the strict definition of "stemness".

Not only do MPCs lose their ability to rapidly proliferate with long term culture, they also appear to lose their "differentiation" and therapeutic potential over

time. Previous studies have shown the *in vitro* differentiation potential of MPCs in humans and other species decreased above passage 30 in osteogenic and adipogenic assays, while telomere length in these cells decreased over time [2-4]. Long term culture also appears to decrease the supportive activity that MPCs can provide for hematopoiesis [5]. Early passages of MPCs were able to promote $CD34^+$ HSC engraftment when co-injected into SCID mice, but beyond passage 9, MPCs were unable to promote HSC engraftment [5]. In addition, MPCs have other potential therapeutic effects which have been shown to decrease with extended culture. In one study, cultured murine MPCs were placed into areas of damage in rat hearts. The murine MPCs lost their protective effects as the culture expansion times before implantation increased from passage 3 to passages 5 and 10. The recipients who received later passage cells had subsequent lack of improved cardiac function compared to rats who received passage 3 cells [6]. Extensive expansion of MPCs appears to decrease their therapeutic usefulness, so most studies recommend limiting *ex vivo* culture to minimize any potential loss of efficacy.

Another problem in classifying MPCs is that the minimal criterion used to define human MPCs does not clarify if the cell surface phenotype refers only to established MPC cultures, or if cells must have the same protein phenotype from initial isolation throughout culture [7]. It is difficult to determine the true lineage of MPCs when these cells appear to undergo dramatic shifts in gene and protein expression in response to environmental cues. For example, when removed from their niche in the bone marrow, putative MPCs are stimulated to rapidly divide, activating proliferation pathways. By definition, a true stem cell should divide infrequently, suggesting that cultured MPCs again do not fit the description of a true stem cell, but may represent a transient amplifying descendant.

I have demonstrated a number of dynamic changes in cell surface molecules on equine bone marrow cells over time in culture. My results suggest that cell surface proteins change in response to the culture environment. For example, cells selected for CD14 cell surface protein at 2 days of culture appear to downregulate expression of this epitope over the next few weeks of culture. CD14 expression is maintained at low levels in long term MPC cultures, suggesting that expression does eventually stabilize unless stimulated by another factor. Stimulation with lipopolysaccharide (LPS) caused a significant upregulation in both *CD14* gene and protein expression, demonstrating that MPCs can produce the LPS receptor, a cell surface protein specifically associated with hematopoietic cells. Other hematopoietic specific cell surface proteins such as CD11a/CD18 are not expressed in bone marrow cells cultured more than two weeks. However, it is still unclear if the protein is not present because the MPC population is truly non-hematopoietic in lineage, or if the expression of CD11a/CD18 has been downregulated as a response to extended culture. Similarly to the previously described LPS stimulation test, CD11a/CD18 expression patterns in MPCs could be tested for gene and protein response in samples via intentional contamination with bacteria, or another known stimulant such as phorbol myristate acetate [8]. This test could help better define the lineage of MPCs as either hematopoietic or non-hematopoietic, as non-hematopoietic cells should not upregulate the specific hematopoietic protein CD11a/CD18.

Further complicating the classification of putative MPCs is the lack of uniformity in cell surface protein expression in freshly isolated bone marrow cells. I have demonstrated that freshly isolated bone marrow cells differ greatly in phenotype from bone marrow cells that have adhered two hours in culture. Exploiting the difference in phenotype by selection of adherent cells for analysis may help to more clearly distinguish the cellular lineage of early equine MPCs.

Sample handling is another factor that can significantly alter the protein expression results of MPCs analyzed using flow cytometry. A dramatic example of this effect was seen when trypsin was used for cell harvest prior to analysis. The decreased mean fluorescence intensity detection of CD44, CD14, and CD172a proteins following trypsinization suggested these molecules were damaged by trypsin. Unfortunately, since the original description by Pittenger et al, many MPC characterization papers use trypsin as part of their protocols for adherent cell harvest prior to analysis by flow cytometry [9]. Although I have not tested the effect of trypsin in MPCs of other species, it is likely that damage to certain epitopes is not a phenomenon unique to the horse. A study by Bryniarski et al. demonstrated that CD14 was trypsin labile in murine cells [10]. Further studies of MPCs in other species would be required, and would be of value to confirm whether damage to the cell surface proteins by trypsin could be a plausible explanation for the apparent discrepancy in MPC phenotype between horses and other species.

What if Putative Equine MPCs are from a Hematopoietic Lineage?

In theory, if putative MPCs are descendants of a hematopoietic lineage precursor, a hematopoietic specific antibody should be capable of enriching the MPC population. Over the course of my research, I have discovered that positive selection using a mouse anti-equine CD14 antibody and magnetic activated cell sorting (MACS) on equine bone marrow cells cultured for 2 days resulted in an enrichment of MPC colony formation. Only certain lineages of hematopoietic cells (e.g. monocytes, macrophages, dendritic cells, activated B lymphocytes and to a lesser extent neutrophils) are known to express CD14 protein on their cell surface. The enrichment of MPC colonies in my study was unexpected based on the CD14 expression results reported in humans and other species for over a decade. Based on previous

information, equine MPC colonies should have been concentrated in the CD14 negative fraction. Cultures of CD14 positively selected cells contained approximately double the number of MPC colonies compared to unsorted bone marrow cells and over twenty times the number of colonies in CD14 negatively selected cells at one week of culture. Two possible interpretations of the dramatic concentration of MPC colony formation in the CD14 positive fraction include 1) MPCs truly lack CD14 expression and are merely using the CD14 positive cells for cell-cell signaling and stimulation of colony formation or 2) MPCs are derived from the CD14 positive fraction. Although possible, the former possibility seems unlikely given the long term low levels of CD14 gene and protein expression detected in cultured MPCs, the up regulation of CD14 expression in MPCs in response to LPS stimulation, and the repeatability (with slightly less efficiency) of MPC colony formation results using a mouse anti-human CD14 antibody for sorting of equine bone marrow cells cultured 2 days (data not shown). If the latter is the cause of MPC enrichment in the CD14 positive fraction, one of the main tenets of previous MPC studies (that MPCs are non-hematopoietic in lineage because they lack hematopoietic markers such as CD14) would need to be reevaluated. A novel approach to further explore the question of whether MPCs are from a hematopoietic origin would be to use other hematopoietic markers, such as anti-equine CD11a/CD18 or MHC Class II antibodies, to sort bone marrow cells cultured 2 days and determine if MPC colony formation is also enriched in the positive fractions.

Ultimately, if putative MPCs are found to be of hematopoietic lineage, their reclassification will not only have an impact on nomenclature, but also on their clinical indications. As will be discussed later in this chapter, if MPCs are descendants of a hematopoietic precursor, the therapeutic goal for their clinical use will need to shift from tissue regeneration to improved tissue repair and function;

however, before this is discussed, the current knowledge of MPC behavior *in vivo* needs to be summarized.

Mesenchymal Progenitor Cell Behavior In Vivo

To date, many studies have investigated the behavior of MPC *in vivo*. Although the initial goals of these studies were to demonstrate tissue regeneration as a direct result of MPC activity, most studies have concluded the main therapeutic effect of these cells is through secretion of trophic factors which modulate the immune response and regenerative microenvironment of the recipient [11-14]. "Differentiation" of MPCs into various mesenchymal tissues *in vitro* has not been repeatable following implantation of MPCs *in vivo.* In fact, no study has been able to definitively demonstrate a tissue regenerative effect *in vivo* using MPCs to form the desired functional tissue. However, a therapeutic effect from MPCs has been evaluated and observed in a variety of diseases including graft versus host diseases, bone disorders, myocardial infarcts, stroke, and spinal cord injuries [11]. Modulation of immune response and growth factor secretion during healing can improve clinical outcome and should be considered as valuable therapeutic effects of MPCs.

In contrast, claiming "tissue regeneration" or "stem cell therapy" in unproven MPC applications might lead to confusion and false hopes and/or expectations for patients. The potential augmentation of healing with use of MPCs in cell therapy could be disappointing to a patient who is expecting tissue regeneration. Patients need to be informed of the potential benefits of MPC therapy, while also being educated to recognize the hype associated with "stem cell therapy". Providing realistic expectations of success to patients will vastly improve the credibility of MPC cell based therapies long term and promote continued evaluation of beneficial MPC properties in future studies.

Application of Findings to Future Equine MPC Studies and Clinical Relevance

MPC-based therapies have been widely used in the horse to treat musculoskeletal conditions ranging from tendon, ligament, and cartilage defects that resulted from developmental abnormalities, traumatic lesions, and degenerative joint disease. Commercial cell-based therapies have utilized cells isolated from various tissues including fat and bone marrow aspirate. Cell processing techniques prior to implantation are also widely variable within the tissue source. For example, cell grafts derived from bone marrow include whole bone marrow aspirate transplant, concentration of the mononuclear cell component of the bone marrow via centrifugation, and implantation of culture expanded putative MPCs. While whole marrow injection requires no additional processing and can be performed patient-side, culture expansion of putative MPCs requires specialized equipment; facilities and many weeks between initial bone marrow aspirate harvest and graft implantation. Clinical results show some benefit in using cultured MPC cell-based grafts, however tissue regeneration does not occur [12].

One major challenge to clinical application of these cells is striking the balance between implanting as many putative MPCs as possible while preventing "terminal differentiation" into a less plastic cell type over prolonged culture. Reducing the time needed between cell harvest and implantation is an important of component of cell-based therapies in the horse. Although putative MPCs derived from CD14 MACS sorting of bone marrow cells are by no means pure at two days of culture, the CD14 positively selected fraction would be a good candidate to compare with other current cell-graft preparations *in vivo*. At two days, cells which form MPC colonies have been concentrated to about 1 in 6,000 cells in the CD14 positively selected fraction. This can be compared to about 1 in 100,000 in uncultured bone marrow mononuclear cells. CD14 positively selected cells at two days of culture should also be relatively

"plastic" compared to bone marrow cells cultured many weeks. My results show no appreciable differences in long term expression of cell surface markers between MPCs derived from unsorted or MACS sorted cells, suggesting that the MPC colonies formed in each fraction have similar properties. Therefore, the clinical benefit of using positively selected cells would be to generate a relatively high concentration of putative MPCs following a relatively short duration of culture.

Ultimately, the search for the cell capable of mesenchymal tissue regeneration must continue. A cell capable of mesenchymal tissue generation must certainly exist to allow for normal tissue turnover over the lifetime of the animal. How can I distinguish the putative MPC population from the cell population which has true tissue regenerative capacity is a question that remains to be explored and answered. Techniques to promote *in vivo* mesenchymal differentiation via cells with induced pluripotency or embryonic stem cells induced down a mesenchymal differentiation pathway will likely be the most promising sources of truly regenerative mesenchymal cells. However, further work to exploit the therapeutic benefits of adult derived cells can be useful, and should not be overlooked as indications for clinical applications. Clearly many questions remain in the relatively poorly understood field of equine MPC biology.

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APPENDIX

List of relevant CD antigens

CD antigen	Alternate name(s)	Distribution
CD3	CD3γ	T, Thymocyte
CD11a	LFA-1, integrin αL	lymph, gran, mono, mac
CD11b	Mac-1, integrin αM	myeloid cells, NK
CD13	Aminopeptidase N	myeloid cells
CD14	LPS-R	Mono, mac, Langer, gran (low)
CD18	integrin β2	hema
CD19	B4	B, FDC
CD29	platelet GP11A, integrin β1	Hema, endo, fibro, mast
CD34	My10, Mucosialin	Hema prec, endo, embryonic fibro
CD44	H-CAM, Pgp-1	Hema and non-hema except plat
CD45	LCA, PTPRC, B220	Hema (multiple isoforms)
CD45RB	LCA	B, T, mono, mac, gran
CD79alpha	Iga, MB-1	B
CD90	Thy-1	CD34+ hema sub, neurons, gran
CD105	Endoglin, SH2	Endo, bone marrow sub, activated mono/mac
CD172a	SIRPα	mono, T, stem cells

Endo	endothelial cells	NK	natural killer cells
FDC	follicular dendritic cells	Mac	macrophages
Fibro	fibroblasts	mono	monocytes
Gran	granulocytes	plat	platelets
Hema	hematopoietic cells	prec	precursors
Langer	Langerhans cells	sub	sub-set
Lymph	lymphocytes		